Universidade de Aveiro Departamento de Biologia
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Fotoinactivação de bactérias bioluminescentes por compostos porfirínicos

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Photoinactivation of bioluminescent bacteria by porphyrinic compounds

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Mestre Eliana Sousa Cruz Ferreira Alves, Bolseira de Doutoramento do Departamento de Biologia da Universidade de Aveiro (SFRH/BD/41806/2007).

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palavras-chave

Terapia fotodinâmica antimicrobiana; porfirinas catiónicas; espécies reactivas de oxigénio; mecanismos de fotoinactivação; bioluminescência; *Escherichia coli*; *Vibrio fischeri*; recuperação bacteriana; resistência bacteriana à fotoinactivação

resumo

A terapia fotodinâmica antimicrobiana representa uma alternativa promissora para inactivar eficientemente microrganismos patogénicos como bactérias, vírus, fungos e protozoários. Esta terapia baseia-se na utilização de um fotossensibilizador, que quando é activado por luz, gera espécies citotóxicas que destroem as células-alvo. A inactivação fotodinâmica das células microbianas pode ocorrer através de dois mecanismos oxidativos: a via do tipo I que envolve reacções de transferência de electrões/átomos de hidrogénio do fotossensibilizador com produção de radicais iónicos e a via do tipo II que envolve a transferência de energia do fotossensibilizador para oxigénio molecular com a subsequente formação de oxigénio singuleto. Tendo em conta os mecanismos de inactivação envolvidos no processo fotodinâmico das células microbianas alvo, o aparecimento de estratégias de resistência à terapia fotodinâmica e a recuperação da viabilidade microbiana parecem ser improváveis. O objectivo deste trabalho foi investigar o(s) mecanismo(s) envolvido(s) na inactivação fotodinâmica da bactéria recombinante bioluminescente *Escherichia coli*, utilizando três porfirinas catiónicas *meso*-substituídas como fotossensibilizadores. Foi também avaliada a possibilidade de recuperação da viabilidade bacteriana após fotoinactivação e a probabilidade de desenvolvimento de resistência bacteriana à terapia fotodinâmica antimicrobiana, utilizando duas bactérias Gram-negativas: *Vibrio fischeri* e *E. coli* bioluminescente. Para investigar o(s) mecanismo(s) de fotoinactivação dos três fotossensibilizadores, foram usados vários inibidores de oxigénio singuleto e de radicais livres. Suspensões de *E. coli* a 10^7 UFC mL^{-1} foram distribuídas em copos esterilizados e adicionadas de porfirina à concentração final de 5.0 µM para os derivados porfirínicos Tetra-Py^+-Me e Tri-SPy^+-Me-PF e 0.5 µM para a porfirina Tri-Py^+-Me-PF. Para testar o mecanismo do tipo I foram usados como inibidores de radicais livres L-cisteína e D-manitol (100 mM). Para testar o mecanismo do tipo II foi usada a azida sódica (100 mM) como inibidor do oxigénio singuleto. As amostras foram expostas durante 270 minutos à luz branca (4 mW cm^{-2}). A recuperação bacteriana foi testada através de um ensaio de fotoinactivação de 270 minutos a luz branca (4 mW cm^{-2}) expondo as as suspensões bacterianas de *V. fischeri* e *E. coli* a 5.0 µM do derivado porfirínico Tri-Py^+-Me-PF e mantendo as amostras no escuro durante uma semana de incubação após o tratamento. Para avaliar o possível desenvolvimento de resistência pelas células bacterianas à terapia fotodinâmica, suspensões bacterianas de *V. fischeri* e de *E. coli* foram expostas a luz branca (4 mW cm^{-2}) durante 25 minutos com 5.0 µM de porfirina Tri-Py^+-Me-PF. Colónias isoladas de células sobreviventes após o primeiro tratamento foram novamente expostas à luz visível usando o mesmo protocolo de irradiação.

Este procedimento foi repetido dez vezes para cada estirpe. Os resultados obtidos neste trabalho indicam que as reacções mediadas pelo oxigénio singuleto (mecanismo do tipo II) têm um papel predominante sobre o mecanismo do tipo I no processo de fotoinactivção da bactéria bioluminescente *E. coli* pelos derivados Tri-Py^{+}-Me-PF, Tetra-Py^{+}-Me e Tri-SPy^{+}-Me-PF. As bactérias inactivadas através da terapia fotodinâmica não recuperaram a sua actividade após uma semana de incubação no escuro. As estirpes *V. fischeri* e *E. coli* não desenvolveram resistência a esta terapia ao fim de dez gerações. Apesar do uso de inibidores representar um método simples e eficiente para determinar qual(is) a(s) via(s) implicada(s) no processo de inactivação fotodinâmica, a escolha dos inibidores deve ter em conta a estrutura química do fotossensibilizador. A ausência de desenvolvimento de resistência bacteriana e a não recuperação da viabilidade bacteriana após uma semana de incubação indica que a terapia fotodinâmica antimicrobiana representa um método adequado para inactivar bactérias de forma eficaz usando Tri-Py^{+}-Me-PF.

keywords

Antimicrobial photodynamic therapy; cationic porphyrins; reactive oxygen species; mechanisms of photoinactivation; bioluminescence; *Escherichia coli*; *Vibrio fischeri*; bacterial recovery; bacterial resistance to photoinactivation

abstract

The antimicrobial photodynamic therapy seems to be a very promising possibility for the efficient inactivation of pathogenic microorganisms and it has been demonstrated in bacteria, virus, fungi and protozoa. The concept of this therapy is that a photosensitizer localized in the target cells, when activated by light, generates cytotoxic species that destroy those cells. Two oxidative mechanisms of photoinactivation are considered to be implicated in the inactivation of the target cells: the type I pathway that involves electron/hydrogen atom-transfer reactions to produce radical ions and the type II pathway that involves energy transfer to produce singlet oxygen from molecular oxygen. Having into consideration the type of damages that occur in the microorganisms after the photoinactivation process, the emergence of strategies of resistance to photodynamic therapy and microbial recovery seem to be unlikely. The aim of this work was to investigate the mechanism(s) involved in the photodynamic inactivation of a recombinant bioluminescent *Escherichia coli*, using three *meso*-substituted cationic porphyrin derivatives as photosensitizers. It was also evaluated in this study the possible recovery of bacterial viability after photodynamic inactivation and the probable development of bacterial resistance to antimicrobial photodynamic therapy using two Gram-negative bacteria: *Vibrio fischeri* and the bioluminescent *E. coli*. To investigate the mechanism(s) of photoinactivation of the three photosensitizers in study, various scavengers of singlet oxygen and of free radicals were used. Suspensions of *E. coli* at 10^7 CFU mL^{-1} were distributed in sterilized beakers and the porphyrins were added to a final concentration of 5.0 µM for the porphyrinic derivatives Tetra-Py^+-Me and Tri-SPy^+-Me-PF porphyrins and 0.5 µM for Tri-Py^+-Me-PF porphyrin. To test type I mechanism L-cysteine (100 mM) and D-mannitol (100 mM) were used as free radical scavengers. To test type II mechanism, sodium azide (100 mM) was used as singlet oxygen quencher. The mixtures were exposed for 270 minutes to white light (4 mW cm^{-2}). The bacterial recovery was tested with a photoinactivation assay of 270 minutes with white light (4 mW cm^{-2}) exposing bacterial suspensions of *V. fischeri* and *E. coli* to 5.0 µM of the porphyrinic derivative Tri-Py^+-Me-PF and maintained the samples in the dark during one week of incubation after treatment. In order to assess the possible development of resistance of bacterial cells after photoinactivation, bacterial suspensions of *V. fischeri* and *E. coli* were exposed to white light (4 mW cm^{-2}) for 25 minutes with 5.0 µM of Tri-Py^+-Me-PF. After the first irradiation period, surviving colonies were reexposed to visible light using the same irradiation protocol.

This procedure was repeated ten times for each strain. The results obtained in this work suggest that singlet oxygen-mediated reactions (type II mechanism) play the most important role on the photoinactivation process of the bioluminescent *E. coli* by Tri-Py^{+}-Me-PF, Tetra-Py^{+}-Me and Tri-SPy^{+}-Me-PF derivatives. The results also show that inactivated bacteria do not recover their viability after photoinactivation and that no resistance to photodynamic therapy appears after ten generations for *V. fischeri* and *E. coli*. Although the use of scavengers represents a simple and an efficient approach to determine which pathway(s) is(are) implicated in the photodynamic inactivation process, the choice of the scavenger must take into account the chemical structure of the photosensitizer. The antimicrobial photodynamic therapy represents an adequate method to inactivate bacteria, since bacterial cells do not recover their viability after one week of incubation nor develop resistance to the photoinactivation process using Tri-Py^{+}-Me-PF.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

°C	Degree celsius
µL	Microliter
µM	Micromolar
$^1O_2$	Singlet oxygen
Amp	Ampicilin
aPDT	Antimicrobial photodynamic therapy
BOHTMPn	2,7,12,17-tetrakis(2-methoxyethyl)-9-*p*-carboxybenzyloxyporphycene
CFU	Colony-forming units
Cm	Chloramphenicol
DBCO	1,4-diazabicyclo-2,2,2-octane
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DP	Deuteroporphyrin
DPiBF	1,3-diphenylisobenzofuran
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EDTA	Ethylenediaminetetraacetic acid
$FMNH_2$	Reduced riboflavin 5'-phosphate
g	Centrifugal force
GlamTMPn	2,7,12,17-tetrakis(2-methoxyethyl)-9-glutaramidoporphycene
Gram (-)	Gram-negative
Gram (+)	Gram-positive
H_2O_2	Hydrogen peroxide
$HO^{-}\cdot$	Hydroxyl radical
HpD	Hematoporphyrin derivate
LBS	Luria bertani broth saline
LPS	Lipopolysaccharides
MB	Methylene blue
MO	Microorganisms
MRSA	Methicillin-resistant *Staphylococcus aureus*
NMR	Nuclear magnetic resonance
$O_2^{-}\cdot$	Superoxide ion
OD	Optical density
PAR	Photosynthetically active radiation
PBS	Phosphate buffered saline

PDI	Photodynamic inactivation
PDT	Photodynamic therapy
PI	Photoinactivation
PS	Photosensitizer
PS^{1}	Photosensitizer ground state
PS^{1*}	Photosensitizer singlet excited state
PS^{3*}	Photosensitizer triplet excited state
RLU	Relative light units
ROS	Reactive oxygen species
rpm	Revolutions per minutes
TBO	Toluidine blue O
Tetra-Py^{+}-Me	5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide
TMPyP	*meso*-tetra (*N*-methyl-4-pyridyl) porphyrin tetra-tosylate
Tri-Py^{+}-Me-PF	5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide
Tri-SPy^{+}-Me-PF	5-(pentafluorophenyl)-10,15,20-tris[2,3,5,6-tetrafluoro-4-(1-methylpyridinium-4-ylsulfanyl)phenyl]porphyrin tri-iodide)
Trp	Tryptophan
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
V. fischeri	*Vibrio fischeri*
W	Watt

CHAPTER 1
INTRODUCTION

PHOTOTHERAPY AND PHOTODYNAMIC EFFECT

The term “phototherapy” describes the use of light in the treatment of diseases (Ackroyd et al., 2001; Moan and Peng, 2003) and its use goes back to the ancient civilizations (Bonnett, 2000). It was used in ancient Egypt, India and China to treat skin diseases such as psoriasis, vitiligo and cancer as well as rickets and even psychosis (Spikes, 1985; Epstein, 1990). The famous Greek physician Herodotus, regarded as the father of heliotherapy, emphasized the importance of sun exposure for the restoration of health. However, it was not until recently that the therapeutics effects of sunlight were widely used in medicine (Ackroyd et al., 2001; Moan and Peng, 2003). In the past two centuries, in France, sunlight was used in the treatment of various conditions, such as tuberculosis, rickets, scurvy, rheumatism, paralysis, edema and muscle weakness (Ackroyd et al., 2001). Phototherapy was further developed by the Danish physician Niels Finsen who, at the turn of the last century, described the successful treatment of smallpox using red light which prevented suppuration of the pustules (Finsen, 1901). Then ultraviolet light was used by Finsen (1901) to treat cutaneous tuberculosis and the use of carbon arc phototherapy was developed for the treatment of this condition. These studies allowed Finsen to be awarded a Nobel Prize in 1903 and to be acknowledged as the founder of modern phototherapy (Ackroyd et al., 2001; Moan and Peng, 2003). In 1950, Richard Cremer introduced the phototherapy as a treatment of jaundice in newborn babies (Cremer et al., 1958).

The term “photodynamic effect” was introduced in 1907 by Von Tappeiner and Jodlbauer and is used to refer to the damage and destruction of living cells or tissues in the presence of a photosensitizer (PS), light and oxygen (Von Tappeiner and Jodlbauer, 1907). The photodynamic effect was initially observed in 1900 by Oscar Raab, a medical student working with the Professor Herman Von Tappeiner (Ackroyd et al., 2001; Bonnett et al., 2006). During the course of his study on the effects of the acridine red dye on *Paramecium caudatum* he discovered that the combination of acridine red and light had a lethal effect on the paramecia and demonstrate that this effect was greater than of either acridine alone, light alone or acridine exposed to light and then added to the paramecia (Raab, 1900; Ackroyd et al., 2001). He also discovered the optical property of acridine red

fluorescence and concluded that it was not the light but rather some product of the acridine that induced *in vitro* toxicity. He suggested that this effect was caused by the transfer of energy from light to the chemical (Ackroyd et al., 2001). The first report of parenteral administration of a PS in humans was in 1900 by the French neurologist Prime who used eosin orally in the treatment of epilepsy and discovered that this induced dermatitis in sun-exposed areas of skin (Prime, 1900). With this discovery, Von Tappeiner and the dermatologist Jesionek used a combination of topical eosin and white light to treat skin tumours leading to the first medical application of an interaction between a fluorescent compound and light (Von Tappeiner and Jesionek, 1903).

PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) is a therapy for cancer and other diseases (Hamblin and Hasan, 2004) and is based on the photodynamic principle. The PDT comprises the use of light (normally visible light and laser light directed via optical fiber) (Shackley et al., 1999), molecular oxygen and a non-toxic photosensitizing agent (given systemically, topically or directly into the organ) able to absorb and transfer the energy of the light to molecular oxygen (Dougherty et al., 1998; Shackley et al., 1999) leading to the formation of highly cytotoxic reactive oxygen species (ROS) (singlet oxygen [$^1O_2$], hydrogen peroxide [H_2O_2], superoxide [$O_2^{-\cdot}$] and hydroxyl radicals [$HO^{\cdot}$]). Such species are able to irreversibly alter the cells' vital constituents resulting in oxidative lethal damage of the target cell (Figure 1) (Wainwright, 1998; De Rosa and Crutchley, 2002). PDT was developed in the 1960's by Lipson and Schwartz at the Mayo Clinic (USA) who observed that injection of crude preparations of hematoporphyrin, a non-pure porphyrin resulting by removal of iron from dried blood by treatment with sulfuric acid (Scherer, 1841), led to fluorescence of neoplastic lesions visualized during surgery. To gain an optimal tumour localization preparation, Schwartz treated hematoporphyrin with acetic acid and sulphuric acid and after neutralization, obtain a porphyrin mixture that he termed “hematoporphyrin derivate” (HpD), which was used by Lipson et al. (Dougherty and Henderson, 1992) for tumour detection. HpD contains several porphyrins, monomers as well as dimers and oligomers (Dougherty et al., 1998). HpD has been partially purified with the less-active

porphyrins' monomers removed, to constitute Photofrin® (Dougherty, 1996), the most widely used PS in clinical PDT. Because of the long-lasting skin phototoxicity of Photofrin®, several PS have been introduced in clinical trials (Gomer, 1991; Pass, 1993). Photofrin® absorbs light only up to about 640 nm. Light at longer wavelengths penetrates farther into tissue and most of the new sensitizers have stronger absorbance at 650–850 nm (Dougherty et al., 1998).

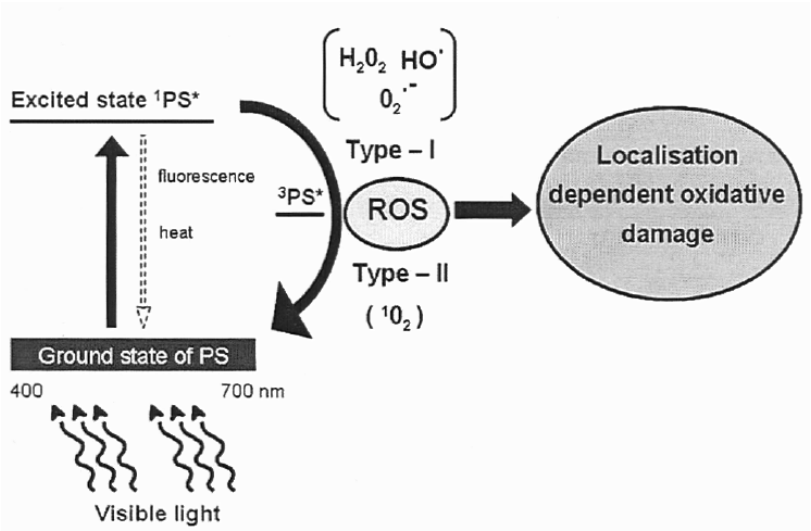


Figure 1 – Principle of photodynamic therapy (Maisch, 2009b).

PDT has the advantage over other therapies of dual selectivity: not only is the PS targeted to the tumour or other lesion, but the light can also be accurately delivered to the affected tissue (Hamblin and Hasan, 2004). The main attraction of PDT is the lack of scarring since connective tissues (including collagen and elastin) tend to be unaffected. The mechanical and functional integrity of the organ is thus left intact (Shackley et al., 1999).

Although originally developed as a cancer treatment, the most successful PDT application to date has been in ophthalmology as a treatment for age-related macular degeneration and has received, in 2000, the USA's Food and Drug Administration approval (Bressler and Bressler, 2000; Hamblin and Hasan, 2004; Wickens and Blinder, 2006). Other non-oncological applications of PDT at a less developed stage include treatments of psoriasis (Boehncke et al., 2000), Barretts's esophagus (Barr, 2000), arthritis

(Trauner and Hasan, 1996), artheroscerlosis (Rockson et al., 2000) and restenosis in both veins and arteries (Jenkins et al., 1999). Besides, resistance to treatment does not seem to develop with repeated use (Lauro et al., 2002). However, photodynamic therapy in its current form has several limitations (Shackley et al., 1999). It is an ablative treatment that yields no biopsy material, so a definitive diagnosis must be made before treatment. It is more complex than other treatment modalities since optimal delivery of light (usually by laser) and drug requires collaboration between scientists and clinicians (Shackley et al., 1999).

ANTIMICROBIAL PHOTODYNAMIC THERAPY

The fact that many human and animal diseases can be caused by microorganisms (MO) has been recognised for many centuries. In the last 150 years, there has been a huge increase in knowledge of the natural history of MO themselves and how they are implicated in the transmission of diseases (Jori and Brown, 2004). The development and widespread use of antibiotics to treat bacterial infections represents one of the most revolutionary advances ever made in scientific medicine. It might have been expected that microbiological-based diseases at the beginning of the twenty first century would have been reduced to a level that no longer had a serious impact on human health (Jori and Brown, 2004). However, the rapid emergence of antibiotic resistance among pathogenic MO may be bringing to an end a period extending over the past 50 years, termed the “antibiotic era” (Yoshikawa, 2002; Hamblin and Hasan, 2004). Bacteria replicate very quickly and a mutation that helps a microbe to survive in the presence of an antibiotic drug will promptly become predominant throughout the microbial population. The problem is further exacerbated by factors of social nature such as the inappropriate or excessive prescription of antibiotics, the failure of some patients to complete their treatment regimen, the more and more frequent transmission of MO due to the global travelling, the expansion of poverty among populations in the third world countries, as well as by the truly large variety of mechanisms adopted by microbial cells to increase their resistance to external insults (Michel and Gutmann, 1997; Hamblin and Hasan, 2004; Jori et al., 2006). These include a thickening of their outer wall, encoding of

new proteins which prevent the penetration of drugs, onset of mutants deficient in those porin channels allowing the influx of externally added chemicals, among others (Harder et al., 1981; Roland et al., 1994; Boyle-Vavra et al., 2001). Due to resistance to all ß-lactam antibiotics, vancomycin, a glycopeptid antibiotic, remained as last line of defense against Gram-positive [Gram (+)] bacteria and methicillin-resistant *Staphylococcus aureus* (MRSA), however vancomycin-resistant *enterococci* are resistant species that are causing much concern at present (Cunha, 1998). The emergence of antibiotic resistance among pathogenic bacteria has led to a major effort to find alternative antibacterial methods, more efficient and faster, non-invasive and non-toxic, which would not lead to microbial resistance (Sommer et al., 2000; Hamblin and Hasan, 2004). One of these non-invasive methods, based on the use of light sources, is the antimicrobial photodynamic therapy (aPDT) (Calin and Parasca, 2009; Maisch, 2009b).

The aPDT seems to be a very promising possibility for the efficient inactivation of pathogenic MO (Wainwright, 1998). The aPDT approach was demonstrated in bacteria, virus and protozoa (Wainwright, 1998; Bonnett, 2000; Wainwright, 2004; Alves et al., 2008; Costa et al., 2008) and is based on the PDT concept: a PS localized in the target cells, when activated by light (visible light as artificial or sunlight), generates cytotoxic species that destroy those cells.

The antimicrobial properties of aPDT have been known for about a century and several studies have shown that antibiotic resistant bacteria are as susceptible to photodynamic inactivation (PDI) as their naive counterparts. The nature of the PDI-induced damage that involves oxidative modification of vital cellular constituents suggests that MO will not easily be able to develop resistance mechanisms (Lauro et al., 2002; Demidova and Hamblin, 2005).

At the moment, the main application of aPDT is in the clinical area for the sterilization of blood and blood products, preventing of viral contamination. Remarkably, the human immunodeficiency virus has been inactivated *in vitro* by aPDT (Wagner et al., 1994; Grandadam et al., 1995; Hirayama et al., 1997; Wainwright, 2000; Ben-Hur et al., 2002; Floyd et al., 2004). Further applications of aPDT would be skin surface disinfection, decolonization of nasal MRSA and treatment of superficial skin wounds (Hamblin and

Hasan, 2004; Maisch et al., 2004). More recently, aPDT was mentioned as a promising alternative to antibiotics to treat oral cavity infections (such as periodontitis, endodontitis, treatment of superificial oropharyngeal *Candida* infections), *Tinea pedis* infection, and acne vulgaris (Jori et al., 2006; Maisch, 2007; Maisch, 2009a). Currently, the aPDT has been studied having in view not only its application to the clinical field, but also to the environmental area (Alouini and Jemli, 2001; Jemli et al., 2002; Bonnett et al., 2006; Jiménez-Hernández et al., 2006; Carvalho et al., 2007; Drábková et al., 2007; Alves et al., 2008; Costa et al., 2008; Carvalho et al., 2009; Oliveira et al., 2009).

This approach has been considered as a possibility to drinking water disinfection process and in wastewater treatment plants as tertiary treatment option in order to overcome the lack of water resources, mainly in densely populated areas, where wastewater may be treated and disinfected to further reuse (Jemli et al., 2002; Rojas-Valencia et al., 2004). Effectiveness of aPDT was observed on the destruction of faecal bacteria (Cerveny et al., 2002; Jemli et al., 2002; Bonnett et al., 2006; Jiménez-Hernández et al., 2006; Carvalho et al., 2007; Alves et al., 2008; Alves et al., 2009) and bacterial endospores (Oliveira et al., 2009), helminths eggs (Alouini and Jemli, 2001) and viruses (Casteel et al., 2004; Costa et al., 2008) in environmental waters. The applicability of the photodynamic principle as a new environmentally-friendly technology to water treatment may become viable if the PS is immobilized on a solid matrix. This solution allows the photoinactivation (PI) process and the subsequent retention of the PS, after photodynamic action, to avoid the release of the PS to the water output (Bonnett et al., 2006; Jiménez-Hernández et al., 2006). As a consequence, some study groups have developed PS immobilized on solid supports and have tested the PI process against faecal bacteria (Bonnett et al., 1997; Artarsky et al., 2006; Bonnett et al., 2006; Jiménez-Hernández et al., 2006; Almeida et al., 2009b; Carvalho et al., 2009). Preliminary results obtained with a *meso*-substituted tricationic porphyrin derivative immobilized in magnetic nanoparticles showed that inorganic-organic hybrids present antimicrobial activity and the fact of the inhert particles having a magnetic core allows the recovery and recycling of the photosensitizing agent (Almeida et al., 2009b; Carvalho et al., 2009). One of the tested inorganic-organic hybrids was able to inactivate Gram (+), Gram-negative

[Gram (-)] bacteria and T4-like bacteriophages to the limits of detection (up to 7 logs of PI) as the PS did in the unbound form (Costa et al., 2008; Almeida et al., 2009b). Besides, Jiménez-Hernandez et al. (2006) used as PS Ru(II) polypyridyl complexes grafted to polymer in a homemade microreactor, with a solar simulator source for laboratory-scale water disinfection assays, using a water sample containing *Escherichia coli* and *Enterococcus faecalis* (2×10^3 CFU mL^{-1}) and obtained a significant photodisinfection with visible light for both microorganisms (Jiménez-Hernández et al., 2006).

Only just a few studies have been made using aPDT for the treatment of fish farms plants. Preliminary results obtained suggest that the photodynamic technique, using porphyrin derivatives as PS, has also a great potential for the disinfection of fish farm waters (Magaraggia et al., 2006; Jori and Coppellotti, 2007; Arrojado, 2009). These studies showed that cell cultures of Gram (+) bacteria (*e.g.*, MRSA), Gram (-) bacteria (*e.g.*, *E. coli*), fungi (*e.g.*, *Candida albicans*) and fungi-like pathogens (*e.g.*, *Saprolegnia* spp.), and parasitic protozoa (*e.g.*, *Acanthamoeba palestinensis*) showed a 5-6 logs decrease in the microbial population after 10 minutes of irradiation with low light intensities (50 mW cm^{-2}) in the presence of micromolar doses of PS (Jori and Coppellotti, 2007). Magaraggia et al. (2006) have also shown that micromolar concentration of a porphyrinic PS promoted the cure of saprolegniosis in trout-farming pools containing naturally or artificially *Saprolegnia* infected fish (inactivation of 6-7 logs at 100 mW cm^{-2} during 20 minutes) without perilesional damage of the fish (Magaraggia et al., 2006). Furthermore, Arrojado (2009) isolated nine bacterial species (*Vibrio anguillarum, Vibrio parahaemolyticus*, *Photobacterium damselae* subsp. *damselae*, *Photobacterium damselae* subsp. *piscicida*, *Aeromonas salmonicida*, *E. coli*, *S. aureus*, *E. faecalis* and *Pseudomonas* sp.) from fish farm water samples and verified that these bacteria were inactivated *in vitro* with a cationic porphyrin (up to 7 logs), at micromolar PS doses, after 90 to 270 minutes of irradiation with a very low light intensity of 4 mW cm^{-2}. It was also determined the impact of aPDT in the total bacterial community of the fish farm water samples. Arrojado (2009) observed that microbial diversity of these waters was also affected by the photodynamic treatment and concluded that it is necessary to treat fish farm waters without the discharge of the water to the environment. These results showed that aPDT can be used to

photoinactivate fish bacterial pathogens in fish farm waters even during dark days of winter time and that more investigation is needed about the impact of aPDT in the environment (Arrojado, 2009).

Irradiation of fish farm waters and wastewaters by solar light, which penetrates deeply into the water column, thereby allowing the uniform illumination of large volumes (Baker and Smith, 1982), makes this technology inexpensive since it is based on the use of free light sources. Moreover, aPDT approach is intrinsically low cost compared to the chemical compounds normally used in aquaculture systems and is conceived to be environmentally-friendly and to exhibit a high level of safety for various ecosystems, as well for humans, animals and plants (Almeida et al., 2009a).

PHOTOSENSITIZERS

A photosensitizer is usually an aromatic molecule that can be a natural or a synthetic compound which undergoes excitation after interaction with an appropriate radiation emitted from a light source (Table 1). This gives rise to activated species which are very reactive towards the chemical environment thus producing molecular damages on important biological targets (Wainwright, 1998).

Table 1 – Photosensitizer absorption maxima (adapted from Wainwright, 1998).

Photosensitizer type	*Wavelength$_{(max)}$ range in buffer (nm)*
Psoralen	300-380
Acridine	400-500
Phenazine	500-550
Cyanine	500-600
Perylenequinonoid	600-650
Porphyrin	400-450
Phenothiazinium	620-660
Phthalocyanine	660-700

A photosensitizing agent with potentially optimal properties should be endowed with specific features, in addition to the expected photophysical characteristics such as a high quantum yield for the generation of both the long-lived triplet state and the cytotoxic singlet oxygen species, a good absorption capacity at the wavelength of the spectral region where the light source is emitted and a good efficiency to generate ROS (Wainwright, 2000; De Rosa and Crutchley, 2002; Jori and Brown, 2004). Such features include (Jori et al., 2006):

- broad spectrum of action, since one PS can act on bacteria, fungi, yeasts and parasitic protozoa;
- efficacy independent of the antibiotic resistance pattern of the given microbial strain;
- possibility to develop PDT protocols which lead to an extensive reduction in pathogen population with very limited damage to the host tissue;
- lack of selection of photoresistant strains after multiple treatments;

- small probability to promote the onset of mutagenicity;
- availability of formulations allowing a ready and specific delivery of the PS to the infected area;
- use of low cost light sources for activation of the photosensitizing agent.

A large number of different compounds with photodynamic activity are now available. First of all the synthetic non-porphyrin compounds have demonstrated photosensitizing ability, like the phenothiazine dyes: methylene blue and toluidine blue. Next, macrocyclic molecules have shown phototoxicity, like phthalocyanines and the metal containing porphyrins as well as the metal-free porphyrins. Another group of dyes belongs to the naturally occurring PS. Psoralens (furanocoumarins) and perylenequinonoids are two examples of natural products which originally act in plants as chemical defence substances against microbial or eukaryotic organisms (Maisch et al., 2004).

It would be desirable to have an effective PS for microbial inactivation without the need of additional chemicals. An important step forward in this direction was prompted by the discovery that PS that are positively charged at physiological pH values such as phenothiazines (Wilson et al., 1995), phthalocyanines (Minnock et al., 1996; Roncucci et al., 1999; Roncucci et al., 2004) and porphyrins (Merchat et al., 1996b) can directly promote the PI of both Gram (+) and Gram (-) bacteria. While phenothiazine derivatives are naturally cationic, owing to the involvement of one amino group in the π electron cloud resonance, porphyrins and phthalocyanines can be transformed into cationic entities trough the insertion of positively charged substituents in the peripheral positions of the tetrapyrrole macrocycle (*meso* positions) and, respectively, tetraazaisoindole macrocycle, which may largely affect the kinetics and extent of binding with microbial cells (Figure 2) (Jori et al., 2006; Magaraggia et al., 2006).

Porphyrins as PS comprise of four pyrrole subunits linked together by four methane bridges (Figure 2). This tetrapyrrole ring structure is named porphin and derivatives of porphins are named porphyrins. Tetrapyrroles are naturally occurring pigments, which are used in many biological processes and include the metallopigments

heme (the prosthetic group of proteins like hemoglobin, cytochromes, catalase, peroxidase and tryptophan pyrrolase), vitamin B12, chlorophyll, siroheme (in nitrite and sulphite reductases) and factor F430 (cofactor of methyl-CoM reductase). All these compounds are synthesized with uroporphyrinogen III as a common intermediate and modified to permit coordination of different metals at the ring centre (iron in heme and siroheme, magnesium in chlorophyll, cobalt in vitamin B12 and nickel in factor F430). These tetrapyrroles do not induce any photochemical or photobiophysical reactions in other compounds or are rapidly quenched in their normal surroundings (*e.g.*, chlorophyll) (Kristian, 2009). The intense Soret band, found around 400 nm, is a characteristic of these systems (Smith, 1975).

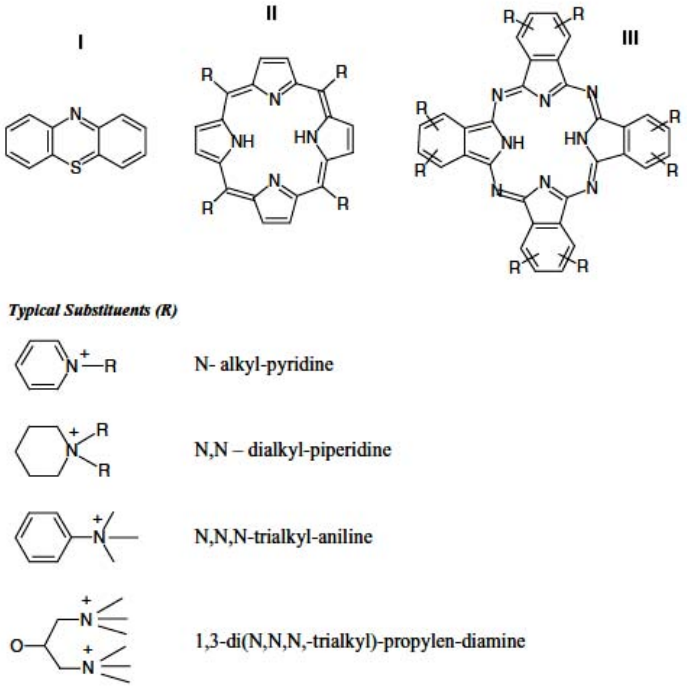


Figure 2 – Basic chemical structure of phenothiazine (I), porphyrin (II), and phthalocyanine (III) photosensitizers and typical peripheral substituents (R) giving the PS a cationic character and enhancing the antimicrobial photosensitizing efficiency (Jori et al., 2006).

As mentioned before, the photodynamic treatment efficiency also depends on several factors such as the presence or absence of charge, charge distribution and the presence of peripheral substituents (Costa et al., 2008; Alves et al., 2009). The porphyrin skeleton is essentially hydrophobic, thus this feature may be an important factor affecting

the preferential accumulation in cellular hydrophobic *loci* since such molecules must be able to get into cells by crossing lipid membranes which brings insolubility in water and physiological fluids (Derycke and De Witte, 2004; Lang et al., 2004; Bautista-Sanchez et al., 2005). The nature and number of the substituents generally have a limited influence on the photophysical properties of the parent compound. However, they may appreciably affect the kinetics and extent binding with microbial cells (Jori, 2006).

Some studies compared the efficiency of synthetic *meso*-substituted cationic porphyrins with different charge distribution (tetra-, tri-, di- or monocationic) (Merchat et al., 1996b; Merchat et al., 1996a; Lazzeri et al., 2004; Caminos et al., 2005; Spesia et al., 2005; Costa et al., 2008; Alves et al., 2009). However, the results achieved are different. Obtained results demonstrated that tetracationic porphyrins are efficient PS against both Gram (+) and Gram (-) bacteria on visible light (Merchat et al., 1996b). Other works demonstrated that some di- and tricationic porphyrins are more efficient than tetracationic ones, both against a Gram (+) strain and two Gram (-) strains (Merchat et al., 1996a) and that a dicationic porphyrin as well as two tricationic porphyrins having a trifluoromethyl group are powerful photosensitizing agents against *E. coli* (Lazzeri et al., 2004). More recently, Alves et al. (2009) investigated the efficiency of seven cationic porphyrins differing in *meso*-substituent groups, in charge number and in charge distribution, on the photodynamic inactivation of a Gram (+) bacterium (*E. faecalis*) and of a Gram (-) bacterium (*E. coli*). The results obtained indicate that the most effective PS against both bacterial strains were the studied tricationic porphyrins followed by the tetracationic porphyrin. However, the dicationic and the monocationic porphyrin derivatives were the least effective ones (Alves et al., 2009). Costa et al. (2008) also tested the same cationic porphyrins on the PI of T4-like sewage bacteriophages and similar results were obtained from those obtained with *E. faecalis* and *E. coli* (Costa et al., 2008). These data indicate that the number of positive charges, the charge distribution in the porphyrins' structure and the *meso*-substituent groups seem to have different effects on the PI of both bacteria and bacteriophages (Costa et al., 2008; Alves et al., 2009).

The nature and distribution of some functional groups in the molecule make it hydrophobic, hydrophilic or amphiphilic. A chemical compound is amphiphilic if it

possesses both hydrophilic and hydrophobic characteristics. In the transport of cell membranes, the lipid bilayer of the membrane allows for passive transport system of hydrophobic molecules. This means that molecules that repel water may diffuse across the cellular membrane without the need for an active transport system. Therefore, the hydrophobic PS can more easily diffuse across the cell membrane and improve the efficiency of photodynamic effect. The hydrophobicity degree of porphyrins can be modulated by either the number of cationic moieties (up to four in *meso*-substituted porphyrins) or by the introduction of hydrocarbon chains of different length on the amino nitrogens (Jori et al., 2006). But, as mentioned, the PS must be in solution, so it needs to be hydrophilic. Then, the ideal PS must have both hydrophilic and hydrophobic proprieties making it amphiphilic. There are some factors which increase the amphiphilic character of the porphyrins: the asymmetric charge distribution at their peripheral positions of the porphyrin, cationic charges combined into different patterns with highly lipophilic groups (*e.g.*, trifluoromethyl groups), the introduction of aromatic hydrocarbon side groups and the modulation of the number of positive charges on the PS (Boyle and Dolphin, 1996; Ando and Kumadaki, 1999; Grancho et al., 2002; Spesia et al., 2005; Banfi et al., 2006; Caminos and Durantini, 2006). The increase in the amphiphilic character of the PS seems to enhance its affinity for MO which helps a better accumulation in the cell/particles (Boyle and Dolphin, 1996; Lazzeri et al., 2004; Spesia et al., 2005) accompanied by an increase in the photocytotoxic activity (Caminos et al., 2005). Besides lipophilicity properties, other important parameters in the make-up of the PS must be considered such as the degree of ionization, electric charge, molecular size and non-specific protein binding (Maisch et al., 2004).

The driving force for binding of the cationic PS to the negatively charged functional groups on the cell surface is of electrostatic nature. Consequently, the binding process is completed within a very short time period. Several independent reports indicate that extending the pre-irradiation incubation from 5 minutes to 1–2 hours has no effect on the amount of PS bound to the microbial cells (Wainwright, 1998; Jori et al., 2006).

PHOTODYNAMIC INACTIVATION OF BACTERIAL CELLS

BACTERIAL DAMAGES INDUCED BY aPDT: SITES OF ACTION AND PHOTOSENSITIVITY

Reactive oxygen species can induce cell damages by the following ways: increasing ion permeability [Na^+/K^+] leakage, loss of repair facility, lysis, inhibition of respiration, inhibition of ribosome assembly, inhibition of replication, base substitution and strand breakage (Wainwright, 1998). Typical type I reactions, for example, at the bacterial cytoplasmatic membrane, include the abstraction of allylic hydrogens from unsatured molecules such as phospholipids. The radical species thus formed may undergo reaction with oxygen to yield the lipid hydroperoxide. Lipid peroxidation is detrimental to membrane integrity leading to loss of fluidity and increased permeability (Korytowski et al., 1992; Wainwright, 1998). Thus inactivation of membrane enzymes and receptors is also possible (Girotti, 1990). The type II reactions are generally accepted as the major pathways in photooxidative microbial cell damage (Wainwright, 1998). Singlet oxygen also reacts with biomolecules involved in the maintenance and structure of the cell wall/membrane such as phospholipids, peptides and sterols (*e.g.*, in yeasts). However, the products from such reactions may be slightly different. Whereas type I reaction with cholesterol may result in the formation of cholesterol-7α or -7β hydroperoxide, the formation of the 5α-isomer is indicative of type II reaction with singlet oxygen (Girotti, 1990). Reactions of singlet oxygen with other molecules involved in the cell wall/membrane also occur (Wainwright, 1998). Nucleic acids are known to react mainly through guanosine residues and again there is a difference in selectivity between type I and type II photoprocesses. The type I reaction is mediated through hydroxyl radical attack at the sugar moiety whereas the type II reaction is an attack of singlet oxygen at guanosine base (Foote, 1990). Some damages produced in the DNA chain can be repaired by the action of DNA repairing systems (Imray and MacPhee, 1973). However, some authors concluded that although DNA damage occurs, it may not be the main cause of bacterial cell death (Hamblin and Hasan, 2004). One argument that has been used in favor of this hypothesis is that *Deinococcus radiodurans*, which is known to have a very efficient DNA repair mechanism, is easily killed by aPDT (Schafer et al., 1998; Maisch, 2009b).

While the predominant type of photodynamic action may often be determined by the class of the compound, the exact mode of action is also closely governed by the site of action. This, in turn, is a function of the physicochemical make-up of the PS (Wainwright, 1998).

Various studies showed that there is a difference in susceptibility to aPDT between Gram (+) and Gram (-) bacteria (Nitzan et al., 1992; Merchat et al., 1996b; Minnock et al., 1996). Anionic and neutral PS were found to bind efficiently to Gram (+) and to induce growth inhibition or killing by aPDT. On the other hand, these PS bind only to the outer membrane of Gram (-) bacteria, being not killed, showing a remarkable resistance to aPDT (Bertoloni et al., 1984; Malik et al., 1990; Bertoloni et al., 1993). The photosensitization by neutral and anionic porphyrins of Gram (-) bacteria is possible only in the presence of membrane disorganizing substances, such as polymyxin B nonapeptide or Tris-EDTA (Nitzan et al., 1992). This is due to the different outer membrane structure of Gram (+) and Gram (-) bacteria (Figure 3). Gram (+) bacteria are characterized by the presence of a 40-80 nm thick outer peptidoglycan wall with no significant amount of lipids or proteins. This murein sacculus contains up to 100 peptidoglycan layers which network does not represent a permeability barrier because it is more or less porous. In contrast, Gram (-) bacteria contain an additional membrane layer in the cell architecture, which is located outside the peptidoglycan layer and shows an asymmetric lipid structure composed by strongly negatively charged lipopolysaccharides (LPS), lipoproteins and proteins with porin function. Hydrophilic compounds can diffuse through the outer membrane using the porins, which are characterized as aqueous channel-forming proteins (Nikaido and Vaara, 1985; Yoshimura and Nikaido, 1985). Therefore, the outer membrane acts as a very effective permeability barrier, making Gram (-) bacteria resistant against host cellular and humoral defense factors. Furthermore, the outer membrane triggers mechanisms of resistance against many antibiotics, which are normally sensitive to Gram (+) bacteria (Nikaido, 1985; Nikaido, 1994).

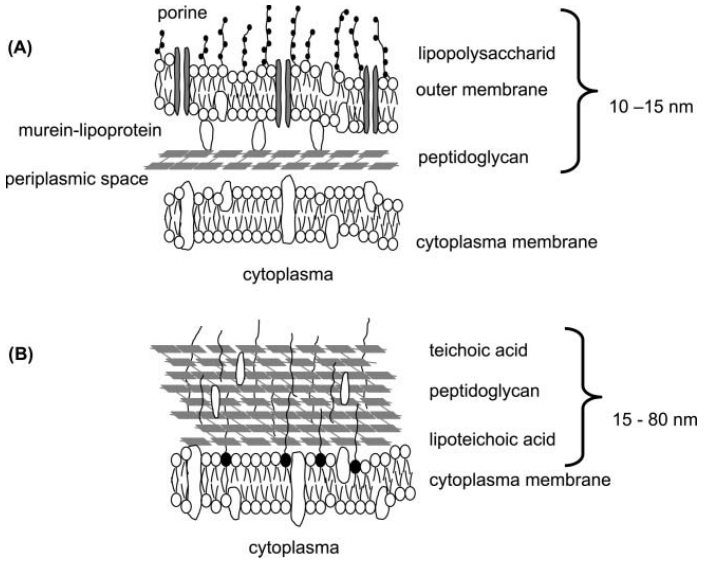


Figure 3 – Schematic representations of the arrangement of the cell wall of Gram (-) (A) and Gram (+) (B) bacteria (Maisch et al., 2004).

In this way, photosensitivity of Gram (-) bacteria with neutral and anionic PS is enhanced by the addition of biological or chemical molecules which alter the native consistence of the outer membrane, thereby enhancing its permeability and facilitating the penetration of phototoxic molecules to the cytoplasmatic membrane (Malik et al., 1992). The addition of Tris-EDTA to Gram (-) bacteria removes the divalent cations (Ca^{2+} and Mg^{2+} ions) which are present in large numbers to stabilise adjacent negative charged LPS molecules at the outer membrane. Then, the neutralization of negative charges is prevented. The onset of electrostatic repulsion promotes the release of up to 50% of the LPS into the medium, thereby allowing the penetration of molecules with the molecular weights as high as 1000 – 2000 Daltons to the inner cytoplasmic membrane or inner cellular compartments (Nikaido and Vaara, 1985). The exposure of Gram (-) bacteria to the action of low concentrations of non-toxic polycations (*e.g.*, polymyxin B nonapeptide or EDTA) displace divalent cationic counter ions because polymyxin B tends to undergo an electrostatic binding with the negatively charged cell surface molecules. By that way, the physical arrangement of the ordered lipid layer is heavily altered with less densely packed hydrocarbon lipid chains (Vaara and Vaara, 1983). As a result, the barrier properties of the outer membrane are strongly reduced and a variety of antibiotics and detergents can diffuse towards the plasma membrane (Ayres et al., 1999).

Cationic PS have several interesting features which make these compounds attractive PS for a variety of biological systems. Studies with cationic porphyrin derivatives have shown to photoinduce direct inactivation of both Gram bacteria without the presence of an additional permeabilization agent (Lazzeri et al., 2004) probably because the cationic sensitizer had a dual action: first in disrupting the bacterial cell wall and then in subsequently photosensitizing the cells (Jori and Brown, 2004). Thus, cationic PS can be considered as the most promising PS for application as broad-spectrum antibacterials (Merchat et al., 1996b; Hamblin et al., 2002; Jori and Brown, 2004; Tomé et al., 2004; Jori et al., 2006).

MECHANISMS OF PHOTOINACTIVATION

The mechanism of cell photoinactivation may be explained as follows (Figure 4): when the PS absorbs light of certain energy it may undergo an electronic transition to the singlet excited state (Ps^{1*}). Depending on its molecular structure and environment, the molecule may then lose its energy by electronic or physical processes, thus returning to the ground state (Ps^{1}), or it may undergo a transition to the triplet excited state (Ps^{3*}) (electron spins unpaired). At this stage, the molecule may again undergo electronic decay back to the ground state, or it can react further by one or both of two pathways know as the type I and type II photoprocesses (Wainwright, 1998; Calin and Parasca, 2009). Type I pathway involves electron/hydrogen-transfer reactions from the PS triplet state (PS^{3*}) with the participation of a substrate to produce radical ions. The type II pathway involves energy transfer from the PS triplet state (PS^{3*}) to molecular oxygen to produce excited-state singlet oxygen ($^{1}O_{2}{}^{*}$). Both of these processes are oxygen dependent and lead to the production of highly ROS that can react with biological molecules causing cell disruption (De Rosa and Bentley, 2000; Hamblin and Hasan, 2004; Donnelly et al., 2008). It is generally accepted that ROS such as $^{1}O_{2}$ and reactive oxygen radicals (H_2O_2, $O_2^{-\cdot}$, $HO^{\cdot}$) generated by the PS action are the species causing cell disruption (Dougherty et al., 1998; Wainwright, 1998; Shackley et al., 1999; Bonnett, 2000; Hamblin and Hasan, 2004).

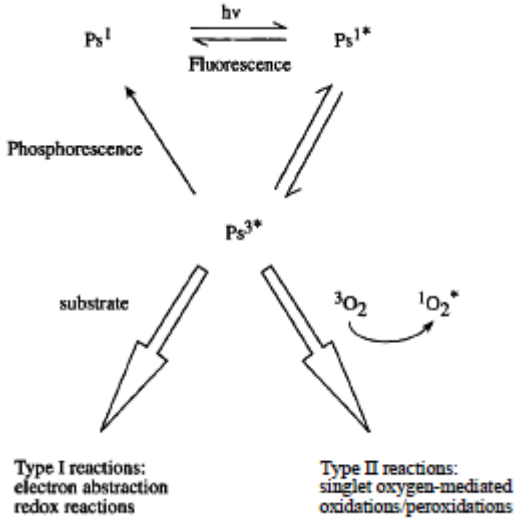


Figure 4 - Photosensitization processes (Wainwright, 1998).

A free radical exists with one or more unpaired electron in atomic or molecular orbital. Free radicals are generally unstable, highly reactive, and energized molecules.

Superoxide anion is a reduced form of molecular oxygen produced by receiving one electron from the molecular oxygen ($O_2 + e^- \rightarrow O_2^{-\cdot}$) (monovalent reduction) (Ochsner, 1997; Harman, 2001). The $O_2^{-\cdot}$ that receives more than one electron and two hydrogen ions forms hydrogen peroxide ($2 \cdot O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$) (Stief, 2003). Superoxide is not particularly reactive in biological systems and does not cause by itself much oxidative damage but it can react with itself by a reaction known as “dismutation” to produce highly reactive oxygen intermediates such as hydrogen peroxide and hydroxyl radicals (Ochsner, 1997).

Hydrogen peroxide is the least reactive molecule among reactive oxygen species that can be generated through a dismutation reaction from superoxide anion by superoxide dismutase. Hydrogen peroxide is highly diffusible and crosses the plasma membrane easily. This ROS is stable under physiological pH and temperature in the absence of metal ions. It is a weak oxidizing and reducing agent and is thus regarded as being poorly reactive (Halliwell, 1997). When H_2O_2 receives more than one electron and one hydrogen ion, the hydroxyl radical is formed ($HO^{-\cdot}$), which is the most reactive of the intermediate ones, once it may react and change any near cellular structure (Jenkins, 1988).

Hydroxyl radical can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as copper or iron ($O_2^{-\cdot} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-\cdot} + O_2$). Hydroxyl radicals have the highest 1-electron reduction potential and can react with everything in living organisms (Korycka-Dahl and Richardson, 1978).

Because of the high reactivity and short half-life of singlet oxygen and hydroxyl radicals, only molecules and structures that are near to the area of its production (areas of PS localization) are directly affected by PDT. The half-life of singlet oxygen in biological systems is inferior to 40 nanoseconds, and, therefore, the radius of the action of singlet oxygen is of the order of 20 nm (Moan and Berg, 1991).

The photodynamic activity to induce cell damage or death is determined by five important photophysical/photochemical properties including (Aveline, 2001):

- an overall lipophilicity and ionization of the photoreactive dyes;
- the molecular exctinction coefficient, ε;
- quantum yield of the triplet state formation, Φ_T;
- redox potentials of the excited states (singlet and triplet) of the PS, if the reaction follows the type I pathway or;
- the quantum yield of the $^1O_2$ generation, if the reaction occurs by a type II photosensitization.

This is important in terms of the preliminary *in vitro* testing of putative compounds: a promising PS in chemical assays may not perform well against its microbial target due to metabolism, reduction and other factors or simply because it localizes in a non-vital region of the target cell. The reverse may also occur: the triphenylmethane dye crystal violet (gentian violet) shows no photosensitizing behavior in chemical tests, yet its inherent bactericidal activity is enhanced by illumination (Wilson et al., 1992). This is thought to result from intracellular adsorption of the PS causing rigidity of its structure and inhibition of the rotational energy loss from the singlet excited state. This leads to increases in the triplet-state yield and thus in the photosensitizing efficacy (Oster, 1955).

METHODS TO DETERMINE THE PHOTOINACTIVATION PROCESS: SINGLET OXYGEN QUENCHERS AND FREE RADICAL SCAVENGERS

It is important to consider some of the strategies to distinguish between type I and type II mechanisms in the PI process. The simplest approach for determining whether $^1O_2$ or another ROS are involved in a photoinactivation process is to investigate the inhibitory effects of various scavengers, *i.e.*, compounds that can intercept these species at high rates and in a putatively selective manner (Girotti, 2001). Some studies were carried out in order to determine which mechanism(s) is(are) responsible for the PI process but only few studies were carried out using MO, namely bacterial cells, and scavengers (Table 2). Others investigations employed chemical techniques without the use of biological systems and/or scavengers (O´Brien et al., 1992; Egorov et al., 1997; Hadjur et al., 1998; Wei et al., 2006; Baier et al., 2007; Maisch et al., 2007; Ribeiro et al., 2007; Magalhães et al., 2008).

Table 2 – Studies using free radical scavengers and singlet oxygen quenchers to determine the photoprocess(es) involved in the inactivation of some microorganisms.

BACTERIA					
Microorganisms	**PS**	**Free radical scavengers**	**Singlet oxygen quenchers**	**Results**	**Reference**
Staphyloccocus aureus	Deutero-porphyrin	Propylgallate	Methionine Tryptophan 1,4-diazabicyclo-2,2,2-octane (DBCO)	Both type I and type II mechanisms	(Nitzan et al., 1989)
Escherichia coli	TiO_2	*Tert*-butanol Methanol	—	Type I mechanism	(Cho et al., 2005)
Escherichia coli	— (UV and simulated sunlight)	Mannitol Superoxide dismutase Cysteine Ascorbate Pyruvate Catalase	Histidine	Type I mechanism	(Khaengraeng and Reed, 2005)
Vibrio vulnificus	Toluidine blue O	Proline	Tryptophan	Both type I and type II mechanisms	(Wong et al., 2005)
Enterococcus hirae *Escherichia coli*	Meso-tetra (N-methyl-4-pyridyl) porphyrin tetra-tosylate	Superoxide dismutase Catalase Dimethyl sulfoxide	Sodium azide Histidine *β*-carotene	Both type I and type II mechanisms	(Ergaieg et al., 2008)
Staphylococcus aureus	— (UV)	Ascorbic acid Catalase Dimethyl-thiourea	—	Type II mechanism	(Maclean et al., 2008)
Staphylococcus aureus	Indocyanine green	—	Tryptophan Deuterium oxide (enhancer of	Type II mechanism	(Omar et al., 2008)

			the life span of $^1O_2$)		
Staphylococcus aureus	Methylene blue	Mannitol	Tryptophan Sodium azide	Type I mechanism	(Sabbahi et al., 2008)
VIRUSES					
Microorganisms	**PS**	**Free radical scavengers**	**Singlet oxygen quenchers**	**Results**	**Reference**
Phages J1, *δ*A, MS2 and *Φ*6	Ascorbic acid	1,2-dihydroxybenzene-3,5-disulfonic acid Potassium bromide Potassium iodide Potassium thiocyanate Sodium formate	1,4-diazabicyclo[2.2.2]octane Sodium azide Guanosine *β*-carotene	Type I mechanism	(Murata et al., 1986)
Herpes simplex virus type I Suid herpes virus type I	3,3'-(1,4-naphthyli-dene)dipro-pionate	Mannitol Glycerol Superoxide dismutase	Imidazol Histidine Sodium azide	Type I and type II mechanisms(mainly type II mechanism)	(Müller-Breitkreutz et al., 1995)
M13 bacteriophage	Metylene blue	(Presence of free radicals determined chemically)	Sodium azide	Both type I and type II mechanisms	(Abe et al., 1997)
T7 phage	5,10,15-(4-h-D-galacto sylphenyl), 20-(2V,3V, 4V,5V-penta-fluorophenyl) porphyrin	1,3-dimethyl-2-thiourea	Sodium azide	Both type I and type II mechanisms	(Egyeki et al., 2003)
MS-2 phage	TiO_2	*Tert*-butanol Methanol		Type I mechanism	(Cho et al., 2005)

The nature of the quenching reaction in a specific PS/quencher pair may vary with the wavelength of photoexcitation, chemical nature of the solvent, oxygen partial pressure and many other physicochemical factors (Wondrak et al., 2005).

Sodium azide and histidine are commonly used to determine the singlet oxygen oxidation of compounds. These agents act as quenchers of singlet oxygen and greatly suppress the activity and consumption of singlet oxygen (Song et al., 1999). Singlet oxygen quenching by sodium azide seems to be a charge transfer process in which molecular triplet oxygen is released after the reaction, therefore no oxygen is consumed (Telfer et al., 1994). The specificity of cholesterol makes its use as an effective indicator of singlet oxygen oxidation, *in situ*, where the use of other detection techniques is difficult (Girotti, 1998; Girotti and Korytowski, 2000). Enhancement of oxygen consumption in deuterium oxide, relative to water, is often used as an indicator of the involvement of singlet oxygen in oxidation due to the increased half-life of singlet oxygen in deuterium oxide (Athar et al., 1988; Pecci et al., 2000). Several other quenching agents have specificity for singlet oxygen: *β*-carotene, imidazole, *α*-tocopherol, tryptophan (Trp), reduced glutathione (Murata et al., 1986; Perotti et al., 2002) and others.

Most free radical scavengers are antioxidants, compounds that neutralize free radicals by donating a hydrogen atom (with its one electron) to the radical. Antioxidants, therefore, reduce free radicals and are themselves oxidized in the reaction (Lieberman and Marks, 2008). Antioxidant enzymes, including superoxide dismutase and catalase, convert free radicals into non-reactive oxygen molecules. Superoxide dismutase converts superoxide anion into hydrogen peroxide and oxygen. Catalase is involved in cellular detoxification and can convert hydrogen peroxide into water and oxygen. Glutathione peroxidase is the most important hydrogen peroxide–removing enzyme existing in the membrane (Papas, 1999). Non-enzymatic antioxidants have a common structural feature: a conjugated double bond system that may be an aromatic ring. Vitamin E (α-tocopherol) is a lipid-soluble antioxidant vitamin that functions is principally to protect against lipid peroxidation in membrane. When vitamin E donates hydrogen to a lipid peroxyl radical, it is converted to a free radical form that is stabilized by resonance. Due to his chemistry, the vitamin E has a greater tendency to donate a second electron and go to the fully

oxidized form (Lieberman and Marks, 2008). Although ascorbic acid is an oxidation-reduction coenzyme that functions in collagen synthesis and other reactions, it also plays a role in free radical defense. Reduced ascorbate can regenerate the reduced form of vitamin E by donating electrons in a redox cycle (Lieberman and Marks, 2008). The antioxidant activity of cysteine is due to its sulphydryl group, acting as the free radical scavenger, and thus as the antioxidants, in biological and other systems (Taylor and Richardson, 1980). Several other quenching agents have specificity for free radicals: melanin (Wang et al., 2008), *tert*-butanol, methanol (Cho et al., 2005), mannitol, glutathione, melatonin (Perotti et al., 2002), dimethyl sulfoxide (DMSO) (Ergaieg et al., 2008), and others.

It is important to determine the PI process of each new PS produced in order to know how the PI occurs in the microbial cell. As the efficiency of microbial PI depends on the amount of singlet oxygen (type II mechanism) and/or of free radicals (type I mechanism) produced during the PI process, it is important to know which of these ROS are generated in order to improve the design of the PS and to improve the design of the photodynamic experiments.

BACTERIAL RESISTANCE TO PHOTOINACTIVATION

Only few studies were done in order to determine if bacterial resistance occurs after various treatments of aPDT. The studies concerning aPDT are more focused on the identification of new PS that kill rapidly and efficiently the MO and to determine the mode of inactivation of the PS. However, and regarding the appearance of bacterial resistance to antimicrobial drugs, it is important to control the process of PI in terms of resistance. Lauro et al. (2002) investigated the selection of resistant bacterial strains in *Peptostreptococcus micros* and *Actinobacillus actinomycetemcomitans* after repeated photosensitization of surviving cells with the porphycene-polylysine conjugates 2,7,12,17-tetrakis(2-methoxyethyl)-9-glutaramidoporphycene (GlamTMPn) and 2,7,12,17-tetrakis(2-methoxyethyl)-9-*p*-carboxybenzyloxyporphycene (BOHTMPn). The results obtained by this group show that the photosensitization of *P. micros* and *A. actinomycetemcomitans* by both GlamTMPn and BOHTMPn induce no appreciable

development of resistance in partially inactivated bacterial cells. In fact, the efficiency of photokilling underwent no change in ten subsequent irradiation sessions, even though cells which were damaged in a previous treatment were cultivated and reexposed to porphycene and light (Lauro et al., 2002). Jori et al. (unpublished data) determined that up to five consecutive generations of extensively photoinactivated MRSA (ca. 90%) show essentially identical degrees of sensitivity to phthalocyanine photosensitization (Jori and Coppellotti, 2007). Moreover, Winckler (2007) affirmed that cell wall structures and membranes are the main target of PDT drugs, and so the drugs do not necessarily need to enter the cell. Specific and proper adhesion to these structures are sufficient for light-activated destruction of the target cell. Thus target cells have no chance to develop resistance by stopping uptake, increasing metabolic detoxification or increasing export of the drug (Winckler, 2007). Various authors refer that resistance to aPDT do not occur after repeated treatments having in consideration for this purpose the damages that can occur in the MO after a process of PI, using none experimental support that tests the efficiency of PI after several treatments (Ito and Kobayashi, 1977; Bagchi and Sreeradha, 1989; Bhatti et al., 1998; Carré et al., 1999; Hamblin and Hasan, 2004; Winckler, 2007; Donnelly et al., 2008; Omar et al., 2008; Cassidy et al., 2009). Bacteria replicate very rapidly and mutations in microbial population occur occasionally. For this reason, it is essential to monitor the PDI process in order to control if mechanisms of resistance occur as a result of microbial evolution and not having only into account the kind of damages that could not induce resistance to aPDT.

RAPID METHODS TO MONITOR THE aPDT PROCESS IN BACTERIA

To monitor the bacterial PI process, faster methods are required instead of the laborious conventional methods of plating, overnight incubation and time-consuming counting of colony-forming units (CFU) (Vesterlund et al., 2004; Demidova and Hamblin, 2005; Alves et al., 2008). New approaches to study potential PS *in vitro* are essential to accelerate the development of aPDT to evaluate which PI pathway is most important and to determine the potential development of mechanisms of resistance.

To this end, the bacterial bioluminescence method is considered to be a rapid (Hamblin et al., 2002), sensitive (Francis et al., 2001) and cost-effective option (Vesterlund et al., 2004). Moreover, it allows only living or viable cells to be detected and does not need exogenous administration of substrates to obtain light emission (Rocchetta et al., 2001).

Bioluminescence refers to the process of visible light emission in living organisms mediated by an enzyme catalyst (Meighen, 1993). The light emission is directly dependent on the metabolic activity of the organism (Vesterlund et al., 2004), once an inhibition of cellular activity results in a decrease in the respiration rate and consequently a decrease in the bioluminescence rate. The phenomenon of bioluminescence has been observed in many different organisms including bacteria, fungi, fish, insects, algae and squid (Meighen, 1993). Luminous bacteria constitute some of the most fascinating subjects in microbiology and are much more prevalent than is frequently appreciated. They are found in marine, freshwater and terrestrial environments (Stewart and Williams, 1992).

The enzymes that catalyze the bacterial bioluminescent reaction are called luciferases and the substrates are fatty acids. Luciferase consists on an oxygenase that catalyzes the emission of light (Ziegler and Baldwin, 1981). The light-emitting reaction involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long chain fatty aldehyde with the emission of blue-green light (Figure 5) (Engebrecht et al., 1983; Rodriguez et al., 1985; Meighen, 1993).

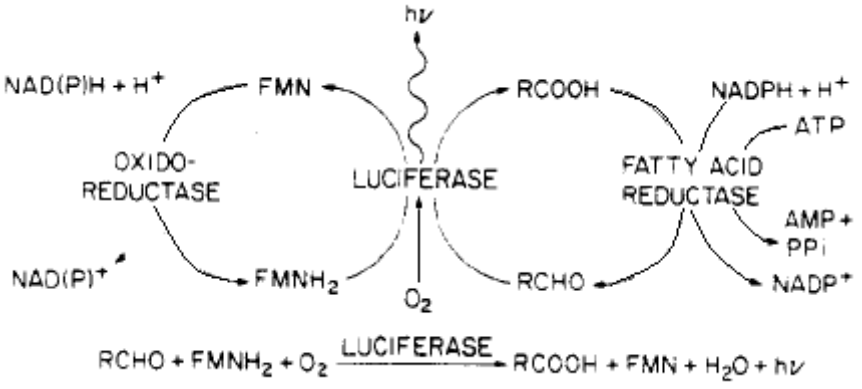


Figure 5 – Substrates, products and pathways involved in the bacterial bioluminescence reaction (Engebrecht et al., 1983).

In both marine and terrestrial bioluminescent bacteria, a five genes operon (*luxCDABE*) encodes the luciferase and biosynthetic enzymes (for the synthesis of the aldehyde substrate) necessary for light production. *luxA* and *luxB* genes encode the alpha and beta subunits of the luciferase, with *luxC*, *luxD* and *luxE* genes encoding proteins for aldehyde production (Meighen, 1991).

The emission of light by most luminescent bacteria is highly dependent on the extent of cellular growth. During the initial stages of growth at low cell density, the *lux* genes are not expressed and luminescence in a cell culture will actually decrease with growth, primary due to a limitation in substrates for the luminescent reaction. During mid to late logarithmic growth, depending on the species and the nutrient composition of growth medium, light emission will increase dramatically. The increase in luminescence arises by activation of expression of the genes in the *lux* operon including the *luxCDABE* genes. Although a number of additional *lux* genes in bioluminescent bacteria have been identified, only *luxCDABE* are essential for the biosynthetic production of light (Meighen, 1993). In marine bioluminescent bacteria light emission occurs preferentially at temperatures below 30°C (Hill et al., 1993).

Light output from these bioluminescent bacteria is a highly sensitive reporter of metabolic activity (Marincs, 2000) and can therefore be used to monitor the real-time effects of antimicrobials on bacterial metabolism (Salisbury et al., 1999; Rocchetta et al., 2001). Moreover, in experimental systems in which a strong correlation between bioluminescence and viable counts can be demonstrated, measurement of bioluminescence offers a rapid, alternative method for monitoring bacterial viability (Marincs, 2000; Rocchetta et al., 2001; Alves et al., 2008). Light output is noncumulative, reflecting actual metabolic rate, and can be measured directly, continuously and non-destructively in high-throughput screening or continuous-culture models (Beard et al., 2002).

With the development of recombinant DNA technology, the phenomenon of bacterial bioluminescence can now be captured and applied within any bacterial species from several rather different perspectives. It provides a real-time non-invasive reporter for measuring gene expression, a sensitive marker for bacterial detection and a measure

of intracellular biochemical function, *i.e.* as a holistic determinant of cellular viability (Stewart and Williams, 1992). Amongst the applications of these recombinant bacteria, the clinical (Contag et al., 2000; Rocchetta et al., 2001; Jawhara and Mordon, 2004; Demidova et al., 2005; Doyle et al., 2006), environmental (Burlage et al., 1990; Ptitsyn et al., 1997; Verschaeve et al., 1999; Johnson, 2005; Grande et al., 2007; Alves et al., 2008) and biotechnological research (Maoz et al., 2002; Kadurugamuwa et al., 2003) are the most promising ones.

OBJECTIVES AND THESIS OUTLINE

The aim of this thesis is to study the mechanism(s) involved in the photodynamic inactivation of bacteria using *meso*-substituted cationic porphyrin derivatives as photosensitizers and a recombinant bioluminescent *Escherichia coli* as the bacterial model in order to use a rapid, sensitive and cost-effective method to monitor the process, instead of conventional laborious methods of dilution, plating and colony counting.
Bacterial viability recovery and resistance studies after aPDT treatments will be also carried out using two Gram (-) bacteria: *Vibrio fischeri* and recombinant bioluminescent *Escherichia coli*.

Chapter 1 consists on a broad introduction, focusing on the photodynamic therapy, the antimicrobial photodynamic therapy, the mechanisms of photodynamic inactivation and the use of a new method to run the aPDT studies.

Chapter 2 describes the work done using the recombinant bioluminescent *E. coli* to determine which photoprocess(es) is(are) implicated in bacterial inactivation by the porphyrin derivatives tested, using different inhibitors of the photodynamic process.

Chapter 3 describes the assessment of bacterial viability recovery and of bacterial resistance to aPDT, using bioluminescent *V. fischeri* and *E. coli* as bacterial models, a *meso*-substituted cationic porphyrin derivative as photosensitizer and artificial white light as light source.

Chapter 4 discusses the obtained results and presents the main conclusions.

CHAPTER 2

MECHANISMS OF PHOTODYNAMIC INACTIVATION OF A GRAM-NEGATIVE RECOMBINANT BIOLUMINESCENT BACTERIUM BY CATIONIC PORPHYRINS

MECHANISMS OF PHOTODYNAMIC INACTIVATION OF A GRAM-NEGATIVE RECOMBINANT BIOLUMINESCENT BACTERIUM BY CATIONIC PORPHYRINS

ABSTRACT

With the emergence of antibiotic resistances by microorganisms it is extremely important to develop alternative treatments for microbial infections. The use of photodynamic therapy as a non-invasive approach to destroy pathogenic microorganisms seems to be very promising. Two oxidative mechanisms are considered to be mainly implicated in the photodamage of cells. In this study, we aim to identify which mechanism(s) is(are) responsible for the photoinactivation of a bioluminescent recombinant *Escherichia coli* by three cationic porphyrins using a rapid method based on the monitoring of the metabolic activity of this bacterium. The inhibitory effect of the photodynamic process was evaluated by exposing bacterial suspensions of 10^5 RLU mL^{-1}, in the presence of singlet oxygen quencher or free radical scavengers, to white light (4 mW cm^{-2}), for 270 minutes, to the photosensitizers (0.5 μM of Tri-Py^+-Me-PF, 5.0 μM of Tetra-Py^+-Me and 5.0 μM of Tri-SPy^+-Me-PF). Sodium azide (100 mM) was used as singlet oxygen quencher, and D-mannitol (100 mM) and L-cysteine (100 mM) were used as free radical scavengers. Tri-Py^+-Me-PF and Tetra-Py^+-Me, in the presence of sodium azide, only led to 0.8 and 1.0 log reduction on *E. coli* bioluminescence, respectively. However, in the presence of L-cysteine and D-mannitol the reduction on bacterial bioluminescence was significantly higher (5.8 and 5.4 log units for Tri-Py^+-Me-PF and 6.0 log units with both scavengers for Tetra-Py^+-Me) when compared with 6.1 and 6.2 log reduction obtained for *E. coli* exposed to Tri-Py^+-Me-PF and Tetra-Py^+-Me without scavengers, respectively. The results obtained with Tri-SPy^+-Me-PF are unexpected and quite different from those with Tri-Py^+-Me-PF and Tetra-Py^+-Me. Reductions of 0.2, 0.7 and 3.2 log units were achieved in the presence of sodium azide, L-cysteine and D-mannitol, respectively. However, the high inhibition of PS activity by L-cysteine is not due to scavenger of free radical but due to a direct inhibition of the PS by the scavenger. In fact, the Tri-SPy^+-Me-PF does not produce $^1O_2$ in the presence of L-cysteine. The results obtained in this study suggest that singlet oxygen (type II mechanism) plays a very important role over free radicals (type I mechanism) on the photoinactivation process of the bioluminescent *E. coli* by Tri-Py^+-Me-PF, Tetra-Py^+-Me and Tri-SPy^+-Me-PF. Although the use of scavengers is an adequate and simple approach to evaluate the relative importance of the two pathways, it is essential to have into account that the scavengers must be chosen having in consideration the structure of the photosensitizer.

Keywords Antimicrobial photodynamic therapy; cationic porphyrins; reactive oxygen species; mechanisms of photoinactivation; bioluminescence; *Escherichia coli*

INTRODUCTION

The emergence of antibiotic resistance amongst pathogenic bacteria is bringing to an end a period extending over the past 50 years (Yoshikawa, 2002). Bacteria replicate very rapidly and a mutation that helps a microbe to survive in the presence of an antimicrobial drug will quickly become predominant throughout the microbial population (Hamblin and Hasan, 2004; Woodford and Ellington, 2007). The inappropriate prescription of antibiotics and the failure of some patients to complete their treatment

regimen also intensify the problem (Hamblin and Hasan, 2004). The resistance mechanisms developed by pathogenic bacteria in the last decades has led to a major effort to find alternative antimicrobial agents, preferentially faster, more efficient, non-invasive and non-toxic to treat microbial infections, which would not lead to microbial resistance (Sommer et al., 2000; Taylor et al., 2002; Hamblin and Hasan, 2004; Jori et al., 2006; Calin and Parasca, 2009).

The use of photodynamic therapy (PDT) as a non-antibiotic approach to inactivate pathogenic MO seems to be very promising (Jori and Perrin, 1985; Konig et al., 2000; Stojiljkovic et al., 2001). Antimicrobial photodynamic therapy (aPDT) approach is based on the PDT concept, in which a photosensitizer (PS) localized preferentially in target cells (*e.g.*, tumour cells, skin cells, MO), when activated by low doses of visible light at an appropriate wavelength, generates cytotoxic species (singlet oxygen and free radicals) that destroy those cells (Wainwright, 1998; Bonnett, 2000; Wainwright, 2000; Maisch, 2009b). Two oxidative mechanisms of inactivation are considered to be implicated in aPDT (Figure 6). After the absorption of the photon by the PS it passes from the ground state (PS^1) to its first excited state (PS^{1*}). From this state, the PS can return again to the ground state (PS^1) or it can pass into a triplet excited state (PS^{3*}). From this triplet excited state, the PS can return to the ground state (PS^1) or it can react further by one or both of the two pathways known as the type I and type II photoprocesses (Calin and Parasca, 2009). Type I pathway involves electron-transfer reactions from the PS triplet state (PS^{3*}) with the participation of a substrate to produce radical ions. The type II pathway involves energy transfer from the PS triplet state (PS^{3*}) to molecular oxygen to produce excited-state singlet oxygen ($^1O_2{}^*$) (Ochsner, 1997; De Rosa and Bentley, 2000; Hamblin and Hasan, 2004; Donnelly et al., 2008). Both of these processes are oxygen dependent and lead to the formation of highly toxic reactive oxygen species (ROS). These ROS can readily react with biological molecules including cholesterol, unsaturated fatty acids in lipid layers of membranes, amino acid residues such as cysteine, histidine, and tryptophan (Trp) of protein structures, as well as nucleic acid bases of DNA, particularly guanine and thymine, and therefore can lead to a loose of appropriate biological functionality producing cell inactivation (Ochsner, 1997; Girotti, 2001; Dolmans et al., 2003).

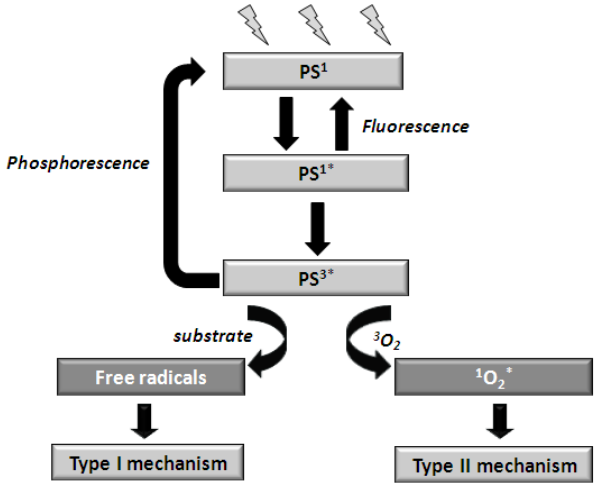


Figure 6 – Photosensitization processes (adapted from Wainwright, 1998).

The use of singlet oxygen quenchers (*e.g.*, sodium azide, histidine, cholesterol, *β*-carotene, imidazole, *α*-tocopherol, Trp and reduced glutathione) and of free radicals scavengers (*e.g.*, superoxide dismutase, catalase, mannitol, glutathione, melatonin, *α*-tocopherol and cysteine) represents a simple approach to determine which pathway(s) is(are) involved in the photodynamic inactivation (Taylor and Richardson, 1980; Murata et al., 1986; Girotti, 1998; Papas, 1999; Song et al., 1999; Girotti and Korytowski, 2000; Girotti, 2001; Perotti et al., 2002; Lieberman and Marks, 2008).

According to the literature, singlet oxygen plays an important role in the photodynamic process and, consequently, the quantum yield of singlet oxygen is a useful parameter to determine the phototherapeutic activity of the PS. Some studies show that type I mechanisms may be equally or even more important than type II pathway. Although these experimental works report which $^1O_2$ or radicals pathways are involved in the photoinactivation (PI) reaction, there are only few studies using MO, namely bacterial cells and scavengers. The inactivation mechanism of *Staphylococcus aureus* by deuteroporphyrin (DP) with singlet oxygen quenchers or hydroxyl radical scavengers was investigated by Nitzan et al. (1989). The light-activated DP (10 μM mL^{-1}) reduced the viability of the culture to less than 1%, whereas methionine, Trp, and 1,4-diazabicyclo-2,2,2-octane (DBCO) used as singlet oxygen quenchers provided almost 60% protection. Propylgallate, which is a hydroxyl free radical scavenger, also provided 60% protection. The presence of a singlet oxygen quencher and propylgallate provided almost complete

protection from inactivation (96%). The results obtained by this group indicate that *S. aureus* PI by DP is mediated by both singlet oxygen and free radicals pathways (Nitzan et al., 1989). Wong et al. (2005) demonstrated that using the free radical scavenger proline and the singlet oxygen quencher Trp, lead to the antibacterial effect of toluidine blue O (TBO) on *Vibrio vulnificus* was markedly reduced indicating that both free radicals and singlet oxygen play an important role on the PI of *V. vulnificus* with TBO (Wong et al., 2005). According to Maisch et al. (2005), when methicillin-resistant *S. aureus* (MRSA) (ATCC BAA-44) was incubated with 0.005 μM of porphyrin XF73 alone or in the presence of 5 mM of the singlet oxygen quencher sodium azide, the survival of MRSA cells increased from 0.015% ± 0.01% to 8.6% ± 0.2% compared to that with incubation with XF73 alone after 15.2 mW cm^{-2} of light irradiation (Maisch et al., 2005). In another study, it was investigated the mechanism of PI of *S. aureus* by methylene blue (MB) using sodium azide and Trp as singlet oxygen quenchers, and mannitol as a hydroxyl free radical scavenger. When the bacterium was treated with MB (20 μM) exposed to 50 mW cm^{-2} of visible light during 10 minutes, it was found that the survival fraction had decreased dramatically to about 31.27 ± 5.39% without quenchers. The presence of sodium azide and Trp failed to show any protection from the MB photodynamic activity. However, in the presence of mannitol, the activity of MB was inhibited, reaching a protection level of about 27%, concluding that the photodynamic activity of MB occurred in part via type I mechanism (Sabbahi et al., 2008). Ergaieg et al. (2008) studied the mechanism involved in the phototoxicity of the cationic porphyrin *meso*-tetra (*N*-methyl-4-pyridyl) porphyrin tetra-tosylate (TMPyP) on *Enterococcus hirae* and on *Escherichia coli* using specific scavengers and quenchers of ROS. They showed that using sodium azide, histidine and *β*-carotene as singlet oxygen quenchers, reductions on the PI activity of *E. hirae* and *E. coli* cells were obtained. Furthermore, using the free radical scavengers superoxide dismutase, catalase and dimethyl sulfoxide (DMSO) they also obtained an important reduction on the activity of TMPyP concluding that both type I and type II reactions play an important role on the PI process of both bacteria using the mentioned porphyrin (Ergaieg et al., 2008).

To monitor the mechanism of bacterial PI, faster methods are required instead of the laborious and time-consuming conventional methods of serial dilutions, plating, overnight incubation and counting of colony-forming units (CFU). New approaches are essential to accelerate the development of aPDT, namely to evaluate which mechanism(s) is(are) responsible for the PI process. The bacterial bioluminescence method, when applied to PI studies to monitor in real-time the bacterial viability, is considered to be a rapid, sensitive and cost-effective option (Hamblin et al., 2002; Demidova and Hamblin, 2005; Alves et al., 2008). In experimental systems, a strong correlation between bioluminescence and viable counts can be demonstrated (Marincs, 2000; Rocchetta et al., 2001; Alves et al., 2008). The light output is noncumulative, reflecting actual metabolic rate, and can be measured directly, continuously and non-destructively in high-throughput screening or continuous-culture models (Beard et al., 2002).

The aim of this study is to identify which mechanism(s) is(are) responsible for the PI of a bioluminescent recombinant *E. coli* using three cationic *meso*-substituted porphyrin derivatives as PS and a rapid detection method based on the metabolic activity of the strain.

MATERIALS AND METHODS

PHOTOSENSITIZERS

The porphyrins used in this work were prepared in two steps according to the literature (Tomé et al., 2004). In the first step, the neutral porphyrins were synthesized by the crossed Rothemund reactions using pyrrole and the adequate benzaldheydes at reflux in acetic acid and nitrobenzene. The resulting porphyrins were separated by column chromatography (silica) and pyridyl groups quaternized by reaction with methyl iodide. Porphyrins were purified by crystallization from chloroform/methanol/petroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. The three PS used were 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py^{+}-Me-PF), 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^{+}-Me) and 5-(pentafluorophenyl)-10,15,20-tris[2,3,5,6-tetrafluoro-4-(1-methylpyridinium-4-ylsulfanyl)phenyl]porphyrin tri-

iodide) (Tri-SPy^{+}-Me-PF) (Figure 7). Stock solutions of 500 µM of all porphyrins (dissolved in DMSO) were prepared and then maintained at 4°C.

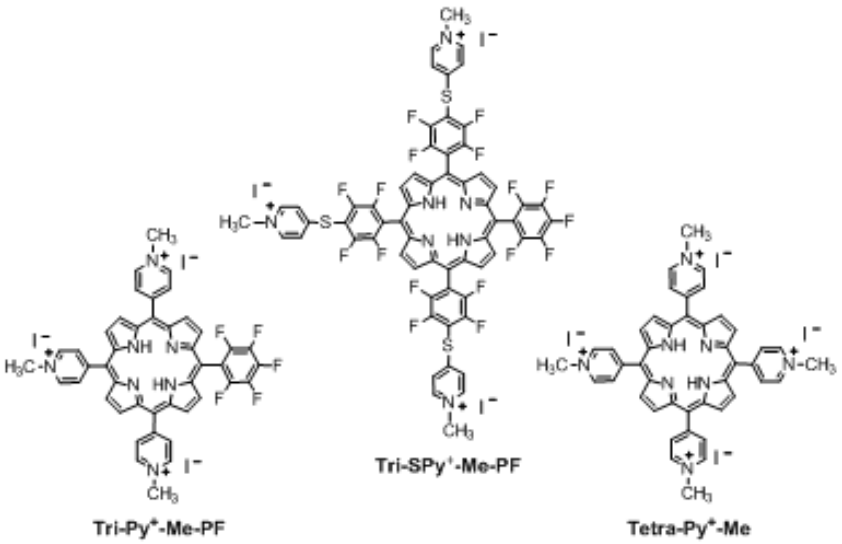


Figure 7 – Structure of the three porphyrin derivatives.

BACTERIAL STRAIN AND BACTERIAL GROWTH CONDITIONS

Bioluminescent *E. coli* was transformed in a previous work and stored at -80°C in 10% of glycerol (Alves et al., 2008). For the transformation, the plasmids pHK724 and pHK555 were inserted into competent cells of *E. coli* Top 10 (Invitrogen, USA), resulting in a bioluminescent strain. These plasmids contain the *lux* operon, required to produce light, from the bioluminescent marine bacterium *Vibrio fischeri* (Alves et al., 2008).

Before each assay, an aliquot of bioluminescent bacteria stored at -80°C in 10% of glycerol was aseptically plated on tryptic soy agar (TSA, Merck) with 100 mg mL^{-1} of ampicilin (Amp) and 25 mg mL^{-1} of chloramphenicol (Cm) and grown for one day at 37°C. Next, one isolated colony was aseptically inoculated on tryptic soy broth (TSB, Merck) with both antibiotics and grown for one day at 25°C under stirring (100 rpm). Afterward, an aliquot of this culture was subcultured in 30 mL of TSB with Amp and Cm and grown overnight at 25°C, under 100 rpm stirring, to reach the stationary growth phase (OD_{600}≈1,3) (Alves et al., 2008).

BIOLUMINESCENCE VERSUS CFU

To evaluate the correlation between the CFU and the bioluminescence signal of *E. coli*, two assays were carried out in dark conditions, with and without porphyrin (Tri-Py^+-Me-PF). Two bacterial suspensions (≈ 10^7 CFU mL^{-1}) were prepared from an overnight culture of bioluminescent *E. coli*, diluting the culture 1:10 in fresh phosphate buffered saline (PBS) 1x (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter; pH 7.4). In one of these bacterial suspensions, the porphyrin (5.0 μM) was added and an incubation was performed in dark conditions during 4 hours at 25-28°C, under stirring. Next, both suspensions were serially diluted (10^{-1} - 10^{-7}) in PBS. Non-diluted (10^0) and diluted aliquots were pour plated on TSA medium (1 mL) and, simultaneously, were read on a luminometer (500 μL) (TD-20/20 Luminometer, Turner Designs, Inc., USA) to determine the bioluminescence signal (measured in relative light units – RLU).

A PI assay was done in order to confirm the correlation between the CFU and the bioluminescence signal. Bacterial cultures grown overnight were diluted in PBS and bacterial suspensions were equally distributed in 600 mL sterilized and acid-washed glass beakers. Then, the appropriate volume of the Tri-Py^+-Me-PF porphyrin was added to achieve the final concentration of 5.0 μM (total volume was 15 mL per beaker). Dark and light controls were carried out during the experiment. The samples were protected from light with aluminum foil and incubated for 10 min under 100 rpm stirring, at 25-28°C, to promote the porphyrin binding to *E. coli* cells. Then, the mixtures were exposed to white light (4 mW cm^{-2}) for 270 minutes (corresponding to a light fluence of 64.8 J cm^{-2}). Aliquots of treated and control samples were collected at time 0 and after 60, 90, 180 and 270 minutes of light exposure and pour plated on TSA. Simultaneously, the aliquots were read on the luminometer to determine the bioluminescence signal.

Both experiments were done in duplicate and the results were averaged.

EXPERIMENTAL SETUP

Cultures of bioluminescent *E. coli* grown overnight in TSB with Amp and Cm were diluted in PBS to achieve a final concentration of 10^7 CFU mL^{-1} (corresponding, approximately, to 10^5 RLU). This bacterial suspension was equally distributed in 600 mL sterilized and acid-washed beakers. Afterwards, the appropriate volume of porphyrin was

added to the respective beaker to achieve the following final concentrations: 0.5 μM of Tri-Py^{+}-Me-PF, and 5.0 μM of Tetra-Py^{+}-Me and Tri-SPy^{+}-Me-PF (total volume was 15 mL per beaker). Then, inhibitors of both PI processes were also added. To test type I mechanism, the free radical scavengers L-cysteine (100 mM) and D-mannitol (100 mM) were used. To test type II mechanism, sodium azide (100 mM) was used as singlet oxygen quencher. Different concentrations of different inhibitors were previously tested and the best concentration was used to study the mechanisms of PI (data not shown). Light and dark controls were included in all experiments. The samples were protected from light with aluminum foil and incubated for 10 min under 100 rpm stirring (25-28°C). Then, the mixtures were exposed to white light (4 mW cm^{-2}) for 270 minutes. Aliquots of treated and control samples were collected at time 0 and after 30, 60, 90, 120, 150, 180, 225 and 270 minutes of light exposure and the bioluminescence signal was measured in the luminometer.

All experiments were done in duplicate and the results were averaged.

IRRADIATION CONDITIONS

The evaluation of inactivation mechanisms of the cationic porphyrins was assessed by exposing bioluminescent *E. coli* in laboratory conditions to white light (PAR radiation, 13 lamps OSRAM 21 of 18 W each one, 380–700 nm) with a fluence rate of 4 mW cm^{-2} (measured with a radiometer LI-COR Model LI-250). As the *V. fischeri lux* genes inserted into *E. coli* make them emit light at temperatures below 30°C (Stewart and Williams, 1992; Hill et al., 1993), the beakers were placed on a tray with water in order to maintain the samples at a constant temperature (25-28°C).

SINGLET OXYGEN GENERATION AND PHOTOSTABILITY STUDIES

Stock solutions of each porphyrin were prepared at 0.1 mM in dimethylformamide (DMF): water (9:1) and a stock solution of 1,3-diphenylisobenzofuran (DPiBF) at 10 mM in DMSO. The reaction mixture of 50 μM of DPiBF and 0.5 μM of the porphyrin derivative in DMF/water (9:1) in glass cells (2 mL) was irradiated with white light, filtered through a cut-off filter of wavelength < 550 nm, at a fluence rate of 9 mW cm^{-2}. During the irradiation period, the solutions were stirred at room temperature. The generation of

singlet oxygen was followed by its reaction with DPiBF. The breakdown of DPiBF was monitored by measuring the decreasing of the absorbance at 415 nm at irradiation intervals of 1 min.

To determine the possible influence of the free radical scavengers L-cysteine and D-mannitol on the activity of the PS tested, singlet oxygen generation assays were done as described before, but adding an appropriate quantity of the scavenger to the PS, from stock solutions of 50 mM. The breakdown of DPiBF was observed by measuring the diminishing of the absorbance at 415 nm at irradiation intervals of 15 to 60 seconds.

The photostability of the photosensitizers was determined by irradiating 2 mL of 1 μM solutions of the porphyrins in DMF/water (9:1) with white light (under the same conditions as those used for the irradiation of the experimental samples). During such irradiation the solutions were magnetically stirred and kept at room temperature. At fixed intervals of time (0; 1; 3; 6; 10; 15; 25; 40; 60; 90; 135; 195; 270 min), the absorbance of the tested porphyrins was determined by visible absorption spectrophotometry at the Soret band.

RESULTS

BIOLUMINESCENCE VERSUS CFU

It was observed a linear correlation between viable counts and the bioluminescence signal of overnight cultures of bioluminescent *E. coli* (Figure 8). This relationship is similar in the absence and in the presence of PS. The correlation obtained between bioluminescence signal and viable counts showed that 10^7 CFU mL^{-1} correspond, approximately, to 10^5 RLU mL^{-1}.

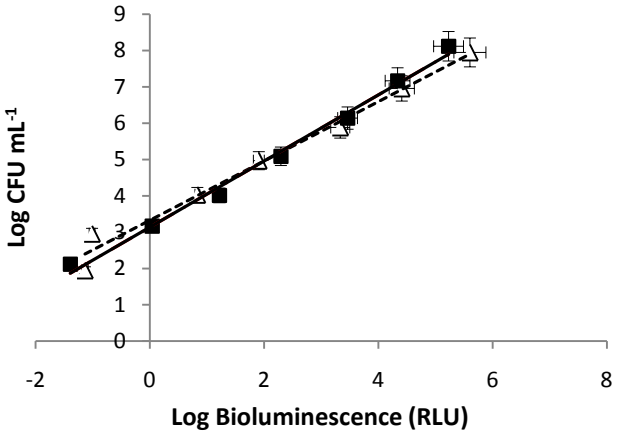


Figure 8 – Relationship between the bioluminescence signal and viable counts of overnight cultures of recombinant bioluminescent *E. coli* serially diluted in PBS. Viable counts are expressed in CFU mL^{-1} and bioluminescence in relative light units (RLU). Each value represents mean ± standard deviation of two independent experiments (--△-- *E. coli* suspension in the absence of PS, —■— *E. coli* suspension with 5.0 µM of Tri-Py^{+}-Me-PF incubated in the dark).

Two experiments of photodynamic inactivation were also done, in order to verify the correlation between the CFU and bioluminescence signal, in the presence of light and PS. *E. coli* cells from overnight cultures were photoinactivated with the *meso*-substitued tricationic porphyrin Tri-Py^{+}-Me-PF resulting in a loss of viable counts (decrease of 7.9 log units) and simultaneously in a loss of bioluminescence signal to the limits of detection (decrease of 5.3 log units) (Figure 9).

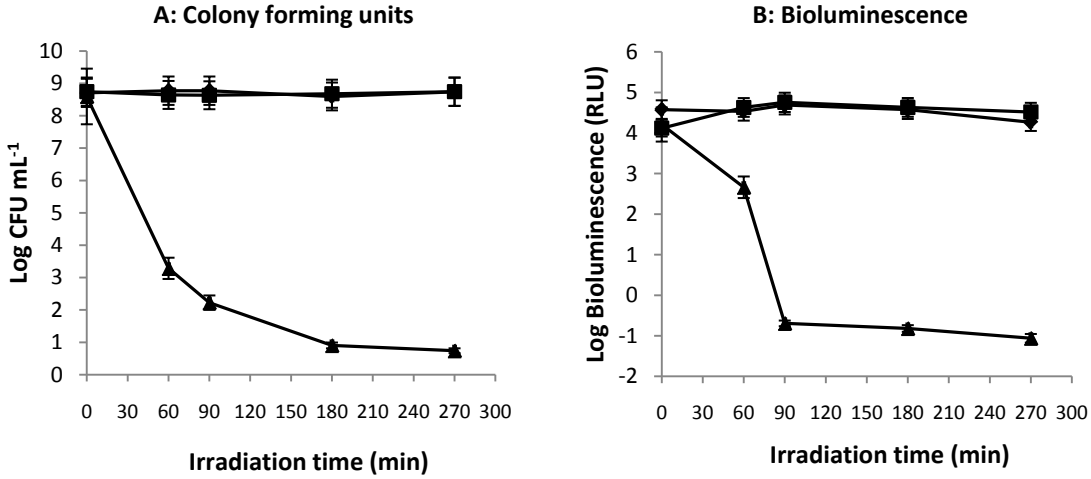


Figure 9 – Photodynamic inactivation assays determined by colony-forming units (A) and bioluminescence (B). Bioluminescent *E. coli* was treated with 5.0 µM of Tri-Py^{+}-Me-PF during 270 min with artificial white light (4 mW cm^{-2}). Each value represents mean ± standard deviation of two independent experiments (-■- Tri-Py^{+}-Me-PF dark control, -◆- *E. coli* light control, -▲- Tri-Py^{+}-Me-PF 5.0 µM).

EFFECT OF FREE RADICAL SCAVENGERS AND SINGLET OXYGEN QUENCHER ON BACTERIAL INACTIVATION

The effect of sodium azide (A), L-cysteine (B) and D-mannitol (C) on the photoinactivation of bioluminescent *E. coli* by Tri-Py^{+}-Me-PF (0.5 µM) is shown in Figure 10. Using sodium azide as singlet oxygen quencher, a reduction of 0.8 log units on bioluminescence signal was obtained after 270 minutes of irradiation, comparatively with a reduction to the limits of detection of 5.9 log units under the same conditions with Tri-Py^{+}-Me-PF (0.5 µM) without scavenger. However, in the presence of the free radical scavengers L-cysteine and D-mannitol the decrease on bacterial bioluminescence was significantly higher, 5.8 and 5.4 log units, respectively (reduction of 6.1 logs with Tri-Py^{+}-Me-PF without scavenger).

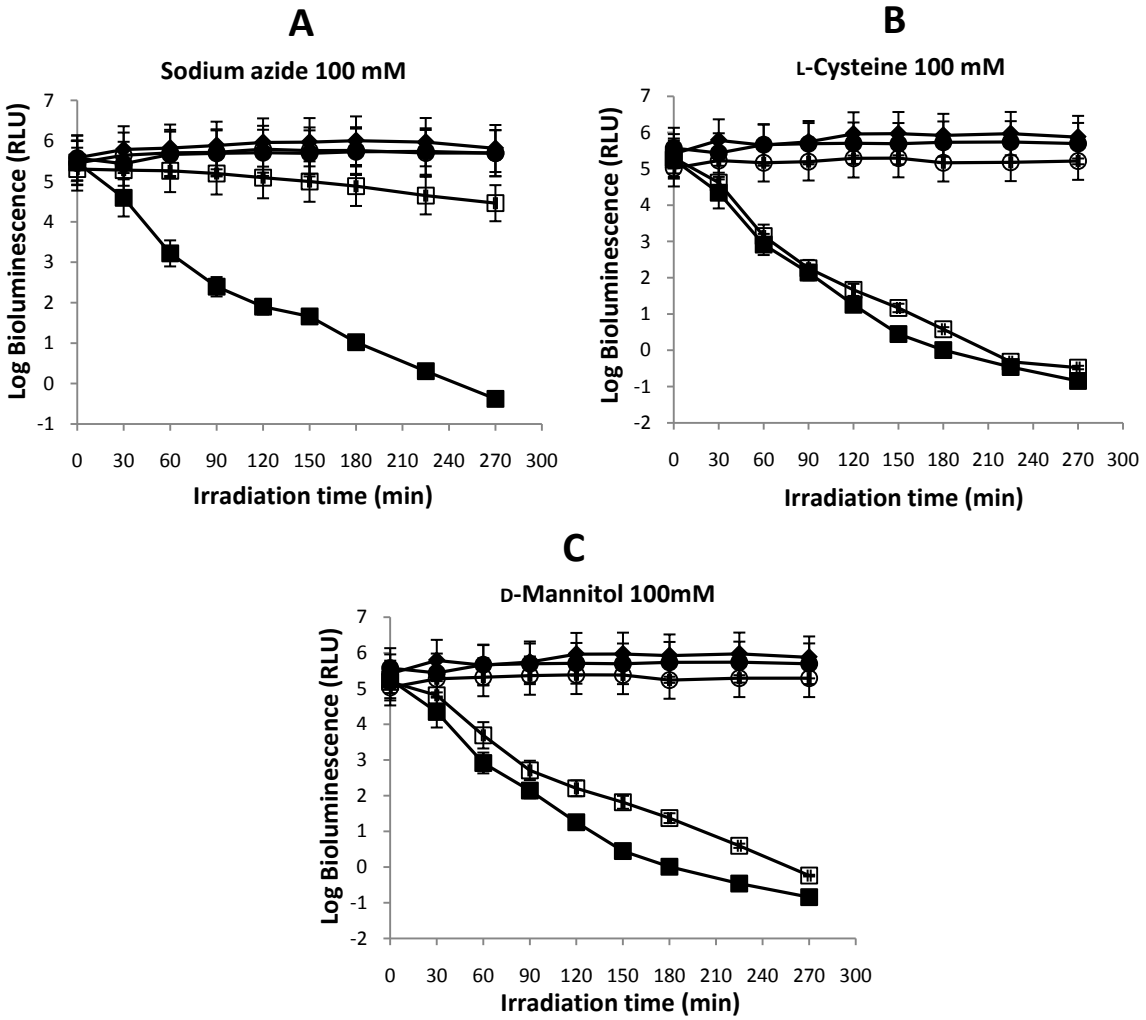


Figure 10 - Effect of sodium azide (A), L-cysteine (B) and D-mannitol (C) at 100 mM on *E. coli* photoinactivation with Tri-Py^{+}-Me-PF. Bioluminescent *E. coli* was treated with 0.5 µM of Tri-Py^{+}-Me-PF during 270 min with artificial white light (4 mW cm^{-2}). Each value represents mean ± standard deviation of two independent experiments (-◆- Tri-Py^{+}-

Me-PF dark control, -■- Tri-Py^{+}-Me-PF 0.5 µM, -□- scavenger 100 mM, -○- scavenger dark control, -●- *E. coli* light control).

The results of sodium azide (A), L-cysteine (B) and D-mannitol (C) on the photoinactivation of bioluminescent *E. coli* by Tetra-Py^{+}-Me (5.0 µM) are represented in Figure 11. In the presence of sodium azide, a reduction of 1 log unit was obtained after 270 min of irradiation comparatively with the control Tetra-Py^{+}-Me (5.0 µM) that has a decrease of 4.9 log units. Reductions of 6.0 log units on bioluminescence signal were achieved with L-cysteine and D-mannitol (reduction of 6.2 logs on bioluminescence signal with Tetra-Py^{+}-Me without scavenger).

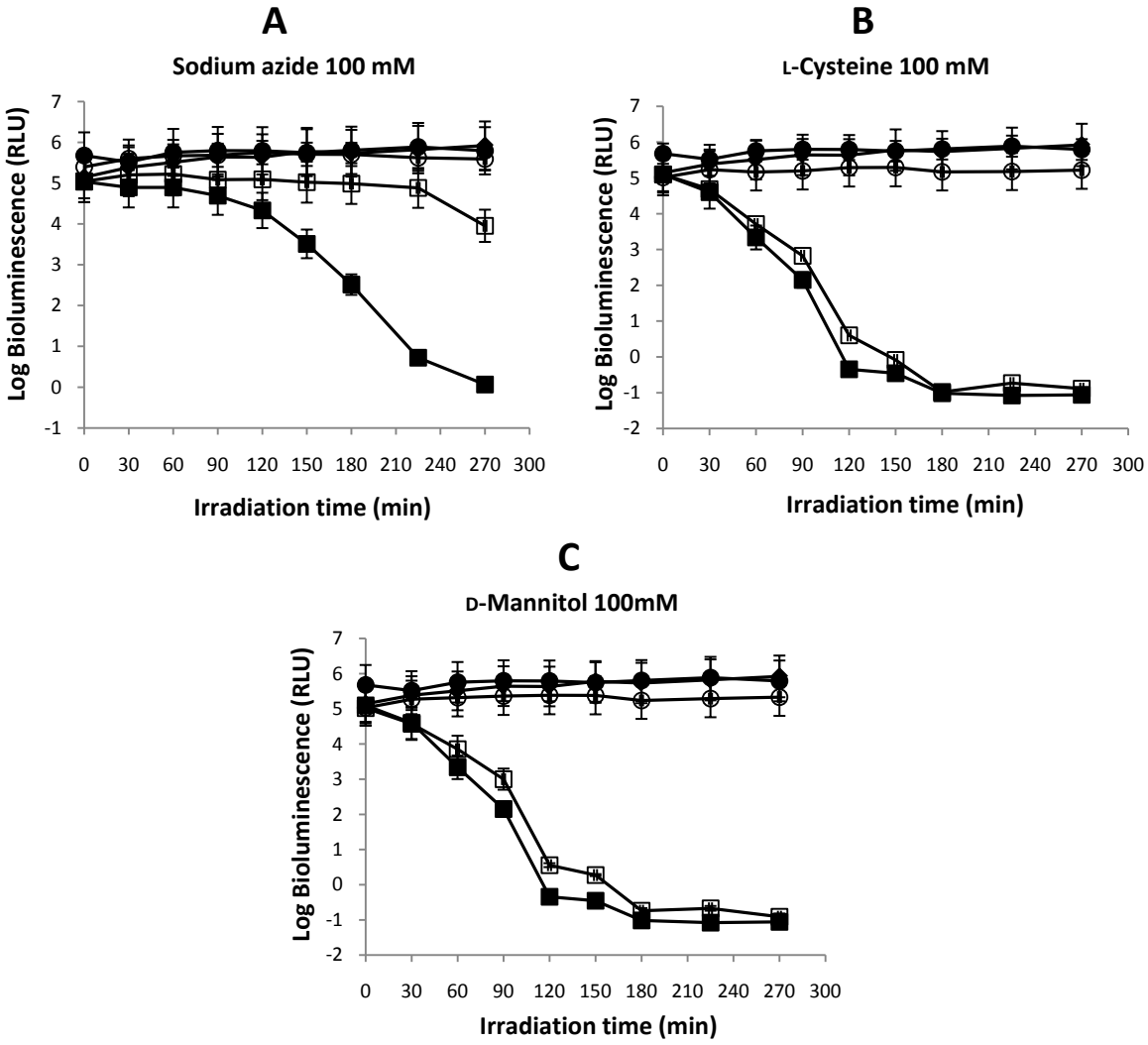


Figure 11 - Effect of sodium azide (A), L-cysteine (B) and D-mannitol (C) at 100 mM on *E. coli* photoinactivation with Tetra-Py^{+}-Me. Bioluminescent *E. coli* was treated with 5.0 µM of Tetra-Py^{+}-Me during 270 min with artificial white light (4 mW cm^{-2}). Each value represents mean ± standard deviation of two independent experiments (-◆-Tetra-Py^{+}-Me dark control, -■- Tetra-Py^{+}-Me 5.0 µM, -□- scavenger 100 mM, -○- scavenger dark control, -●- *E. coli* light control).

The effects of sodium azide (A), L-cysteine (B) and D-mannitol (C) on the photoinactivation of bioluminescent *E. coli* by Tri-SPy^{+}-Me-PF (5.0 μM) are shown in Figure 12. The results obtained with this PS are quite different from those of Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me. After 270 minutes of irradiation, a reduction of 0.2 log units was achieved in the presence of sodium azide (reduction of 3.5 log units with Tri-SPy^{+}-Me-PF without sodium azide) comparatively with 3.2 log units obtained with D-mannitol (reduction of 3.9 log units with Tri-SPy^{+}-Me-PF). However, using L-cysteine as free radical scavenger the results were unexpected. A reduction of 0.7 log unit was achieved comparatively with a reduction of 3.9 logs on bioluminescence signal with Tri-SPy^{+}-Me-PF, after 270 minutes of irradiation.

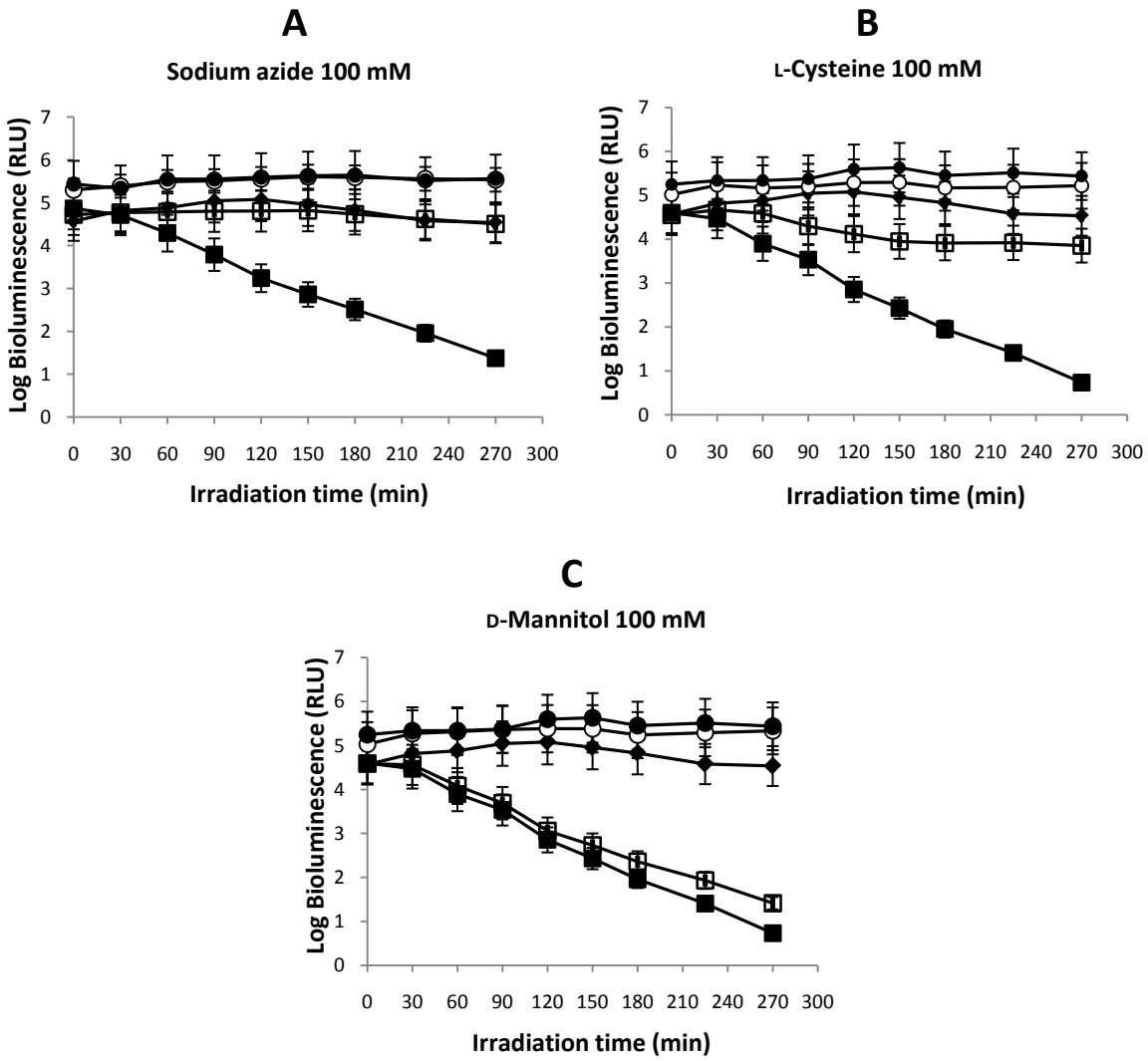


Figure 12 - Effect of sodium azide (A), L-cysteine (B) and D-mannitol (C) at 100 mM on *E. coli* photoinactivation with Tri-SPy^{+}-Me-PF. Bioluminescent *E. coli* was treated with 5.0 μM of Tri-SPy^{+}-Me-PF during 270 min with artificial white light (4 mW cm^{-2}). Each value represents mean ± standard deviation of two independent experiments (-◆- Tri-SPy^{+}-

Me-PF dark control, -■- Tri-SPy$^+$-Me-PF 5.0 µM, -□- scavenger 100 mM, -○- scavenger dark control, -●- *E. coli* light control).

CONTROL SAMPLES

The results suggest that the viability of the recombinant bioluminescent *E. coli* is not affected either by irradiation itself (*E. coli* light control) or by any of the PS tested in the dark (porphyrin dark control), the inhibitors tested in the dark (inhibitor dark control) and inhibitors exposed to light (inhibitor light control) (Figure 13).

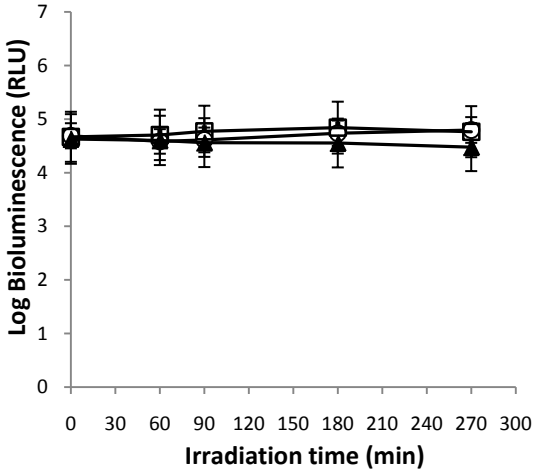


Figure 13 – Light controls of oxygen scavengers: sodium azide, L-cysteine and D-mannitol. Each value represents mean ± standard deviation of two independent experiments (-○- sodium azide light control, -□- L-cysteine light control, -▲- D-mannitol light control).

SINGLET OXYGEN GENERATION AND PHOTOSTABILITY STUDIES

Studies concerning the photostability of the PS were performed and the results are presented in Figure 14A. The results show that Tri-Py$^+$-Me-PF, Tetra-Py$^+$-Me and Tri-SPy$^+$-Me-PF do not photobleach during 270 minutes of irradiation.

The ability of these cationic porphyrins to generate singlet oxygen is represented in Figure 14B. The results indicate that the DPiBF photodegradation is highly enhanced in the presence of the PS. Under the same experimental conditions, the porphyrin Tri-Py$^+$-Me-PF seems to be more efficient than Tetra-Py$^+$-Me which is considered a good singlet oxygen producer (Merchat et al., 1996b; Merchat et al., 1996a; Jemli et al., 2002). On the other hand, the porphyrin Tri-SPy$^+$-Me-PF is the derivative that generates less singlet oxygen.

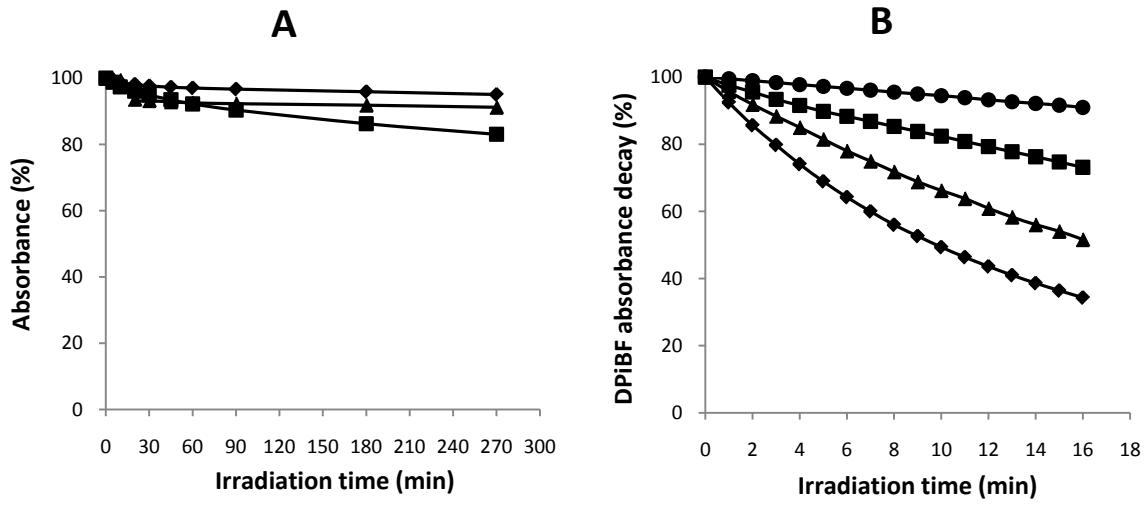
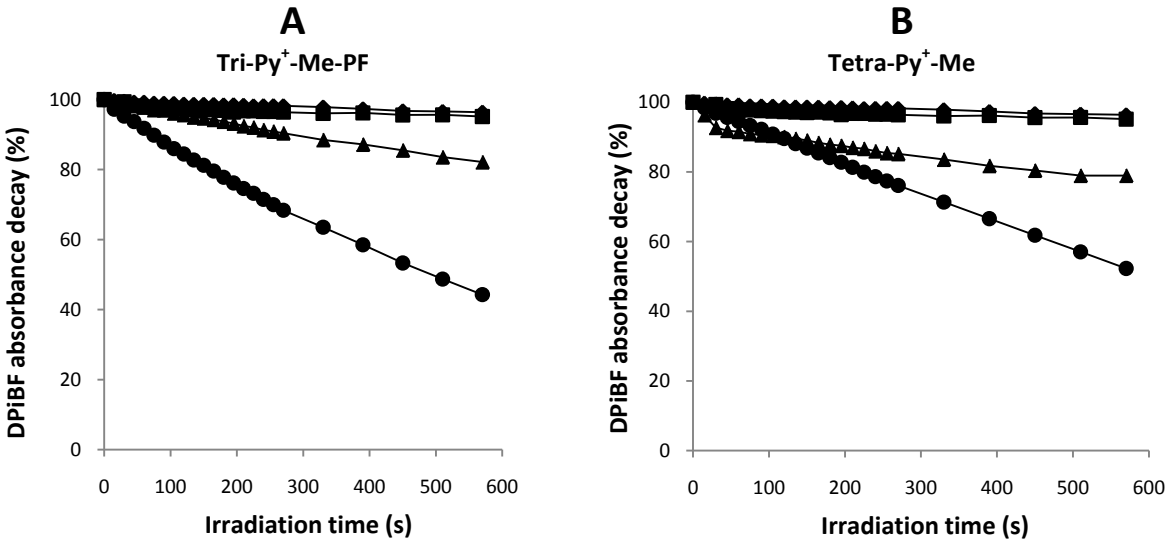


Figure 14 – Photostability of Tri-Py$^+$-Me-PF, Tetra-Py$^+$-Me and Tri-SPy$^+$-Me-PF after irradiation with white light at a fluence rate of 4 mW cm^{-2} (A) (-◆- Tri-Py$^+$-Me-PF; -▲- Tetra-Py$^+$-Me; -■- Tri-SPy$^+$-Me-PF). Photodecomposition of DPiBF by singlet oxygen generated by Tri-Py$^+$-Me-PF, Tetra-Py$^+$-Me and Tri-SPy$^+$-Me-PF after irradiation with white light filtered through a cut-off filter for wavelength < 550 nm (9 mW cm^{-2}) (B) (-◆- Tri-Py$^+$-Me-PF; -▲- Tetra-Py$^+$-Me; -■- Tri-SPy$^+$-Me-PF; -●- DPiBF).

Figure 15 shows the influence of L-cysteine on the generation of singlet oxygen by the porphyrins Tri-Py$^+$-Me-PF (A), Tetra-Py$^+$-Me (B) and Tri-SPy$^+$-Me-PF (C). It can be observed reductions of 40% and 20% on the generation of singlet oxygen when the scavenger was added to the porphyrin derivatives Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me, respectively. In the case of Tri-SPy$^+$-Me-PF, when L-cysteine was added to the PS, it was not observed any generation of singlet oxygen by this porphyrin.



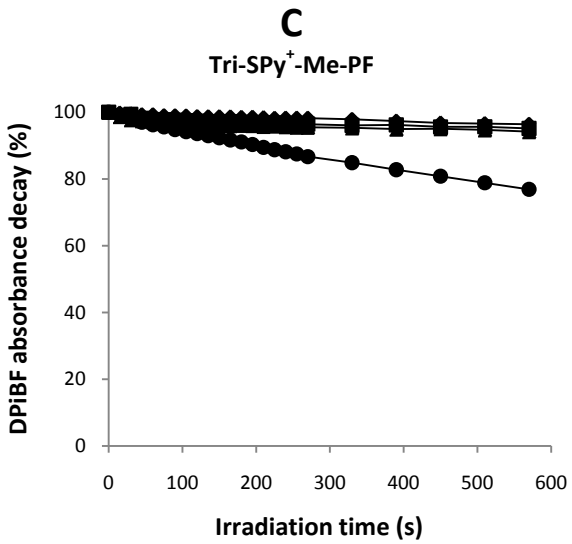
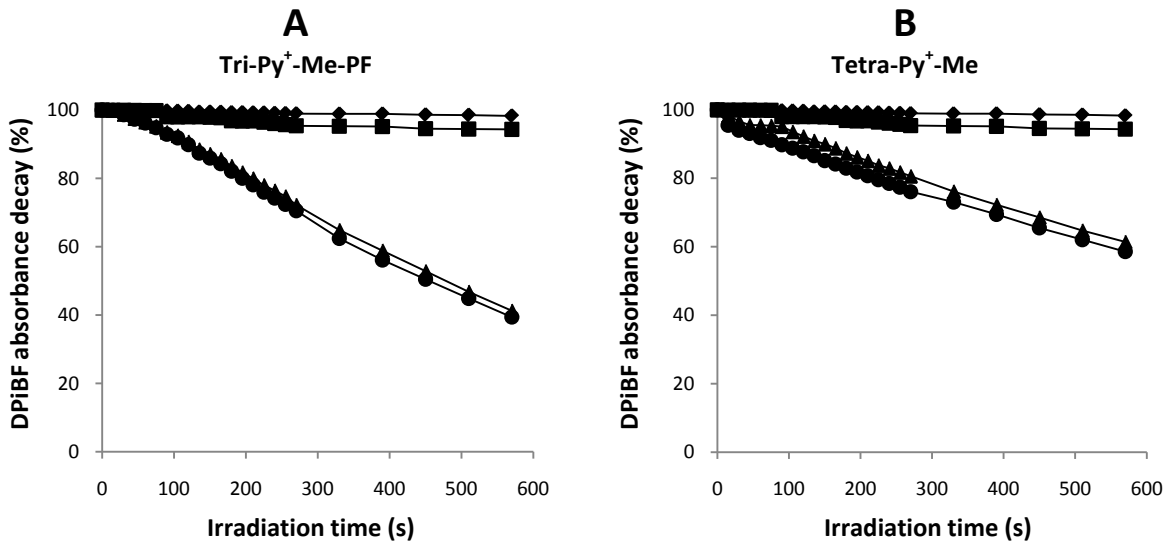


Figure 15 - Photodecomposition of DPiBF by singlet oxygen generated in the presence of L-cysteine by Tri-Py$^+$-Me-PF (A), Tetra-Py$^+$-Me (B) and Tri-SPy$^+$-Me-PF (C) after irradiation with white light filtered through a cut-off filter for wavelength < 550 nm (9 mW cm^{-2}) (-◆-DPiBF; -▲- L-cysteine + PS; -■- L-cysteine; -●- PS).

The influence of D-mannitol on the generation of singlet oxygen of the porphyrins Tri-Py$^+$-Me-PF (A), Tetra-Py$^+$-Me (B) and Tri-SPy$^+$-Me-PF (C) is shown in Figure 16. It was observed that adding D-mannitol to the PS, the obtained data are equal from those obtained with the PS alone, where it was achieved a decay on absorbance of DPiBF of 60%, 40% and 20% by Tri-Py$^+$-Me-PF, Tetra-Py$^+$-Me and Tri-SPy$^+$-Me-PF, respectively.



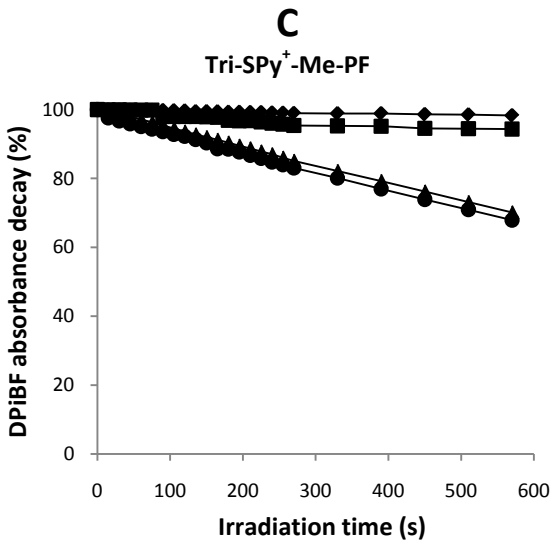


Figure 16 - Photodecomposition of DPiBF by singlet oxygen generated in the presence of D-mannitol by Tri-Py$^+$-Me-PF (A), Tetra-Py$^+$-Me (B) and Tri-SPy$^+$-Me-PF (C) after irradiation with white light filtered through a cut-off filter for wavelength < 550 nm (9 mW cm^{-2}) (-◆-DPiBF; -▲- D-mannitol + PS; -■- D-mannitol; -●- PS).

DISCUSSION

Antimicrobial photodynamic therapy represents a new promising approach to inactivate bacteria, fungi, protozoa and viruses (Wainwright, 1998; Bonnett, 2000; Alouini and Jemli, 2001; Wainwright, 2004; Lambrechts et al., 2005a; Alves et al., 2008; Costa et al., 2008; Oliveira et al., 2009). As the efficiency of microbial PI depends on the amount of singlet oxygen (type II mechanism) and/or of free radicals (type I mechanism) produced during the photodynamic process, it is important to know which of these ROS are generated in order to improve the design of the PS and to decide about the best conditions for microbial PI. The simplest approach for determining whether $^1O_2$ or free radicals are involved in a PI reaction is to study the inhibitory effects of various scavengers of $^1O_2$ and of free radicals (Girotti, 2001).

Tri-Py$^+$-Me-PF porphyrin was recently described as a promising PS for the inactivation of several types of MO (Carvalho et al., 2007; Alves et al., 2008; Costa et al., 2008; Alves et al., 2009; Oliveira et al., 2009). While it was used a concentration of 5.0 µM for Tetra-Py$^+$-Me and Tri-SPy$^+$-Me-PF porphyrins, for Tri-Py$^+$-Me-PF porphyrin it was used a lower concentration (0.5 µM) in order to observe an inhibitory effect of the PI process.

The results of this study, as those obtained in other studies (Müller-Breitkreutz et al., 1995; Hadjur et al., 1998; Maisch et al., 2005; Maclean et al., 2008; Omar et al., 2008),

show clearly that singlet oxygen-mediated reactions (type II mechanism) represent the main pathway through which the PS exert their photodynamic action. However, although singlet oxygen plays the major role in the photodynamic process, free radicals-mediated reactions are produced simultaneously, contributing also to the bacteria PI, however, to a much smaller extent.

The singlet oxygen quencher tested, sodium azide, led to an inhibition of the PI of the three porphyrins used (reductions of 5.1, 3.9, and 3.3 log units on the PI of Tri-Py^+-Me-PF at 0.5 μM, Tetra-Py^+-Me at 5.0 μM and Tri-SPy^+-Me-PF at 5.0 μM, respectively), and this reduction is proportional to the singlet oxygen generation by the PS. Tri-Py^+-Me-PF is the PS that generates the highest amount of $^1O_2$ and it is the porphyrin that shows the highest reduction of PI in the presence of sodium azide. Tri-SPy^+-Me-PF is the porphyrin that generates the smallest amount of $^1O_2$ and is also the PS that shows the lowest reduction of PI in the presence of the scavenger. Moreover, the most effective PS to inactivate Gram-positive and Gram-negative bacteria at 5.0 μM is Tri-Py^+-Me-PF, followed by Tetra-Py^+-Me and Tri-SPy^+-Me-PF (Alves et al. 2009; Alves et al., unpublished data) and the same pattern of variation is observed relatively to the $^1O_2$ production.

The free radical scavenger D-mannitol led to a small decrease on the PI of the three porphyrins (reductions of 0.7, 0.2, and 0.7 log units on the activity of Tri-Py^+-Me-PF at 0.5 μM, Tetra-Py^+-Me at 5.0 μM and Tri-SPy^+-Me-PF at 5.0 μM, respectively). When L-cysteine is used as free radical scavenger, the decrease on the PI of Tri-Py^+-Me-PF and Tetra-Py^+-Me is similar to that obtained with D-mannitol (reductions of 0.3 and 0.2 log units, respectively). However with Tri-SPy^+-Me-PF the inhibition on the PI of the PS is higher (reduction of 3.2 log units), reaching reduction values only a little bit lower than those observed with the $^1O_2$ quencher sodium azide. However, the reduction of PI of the PS by L-cysteine is not due to the inhibition of free radicals produced but due to a direct inhibition of the PS by L-cysteine. The antioxidant activity of cysteine is due to its sulfhydryl group, acting as the free radical scavenger, and so as the antioxidants, in biological and other systems (Taylor and Richardson, 1980). According to the chemical structure of Tri-SPy^+-Me-PF, it is suspected that disulfide bridges take place between L-cysteine and Tri-SPy^+-Me-PF stopping the activity of the PS and, consequently, the

production of ROS. This can explain the fact that using L-cysteine as a free radical scavenger there is a reduction on the bioluminescence signal of *E. coli* as a result of the lost of the activity of Tri-SPy$^+$-Me-PF. In fact, the production of $^1O_2$ by the porphyrin Tri-SPy$^+$-Me-PF in the presence of L-cysteine decreases greatly. Although non-expected, L-cysteine also affects the production of $^1O_2$ by the other two porphyrins without sulfhydryl group. However, the production of singlet oxygen is not stopped by this scavenger as occurred with Tri-SPy$^+$-Me-PF. Besides, these reductions on the generation of singlet oxygen by Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me porphyrins are not relevant to the results since the data obtained with these porphyrins do not exhibit the same result obtained with Tri-SPy$^+$-Me-PF when the free radical scavenger L-cysteine was added to the beaker, even for Tri-Py$^+$-Me-PF that was used ten time less concentrated that the other two PS. It was also tested the effect of D-mannitol on the generation of singlet oxygen and it was verified that this scavenger does not affect the production of $^1O_2$ by all the PS tested.

Porphyrins can undergo photobleaching when exposed to light and oxygen (MacRobert et al., 1989). The rate of photodegradation shown by a PS after exposure to light is an important parameter to assess, because a rapid photobleaching would cause a decrease on the concentration of the PS, thus impairing the efficiency of the treatment (Silva et al., 2005). It was observed that Tri-Py$^+$-Me-PF, Tetra-Py$^+$-Me and Tri-SPy$^+$-Me-PF do not photobleach during 270 minutes of irradiation. Such absence of photodegradation suggests that the concentration of the PS may be unaffected by light during the irradiation period required for the PI of bacteria.

Photosensitized oxidation may change the type of pathways during the course of the reaction as the concentration of compounds and oxygen changes (Min and Boff, 2002). For the conditions used in this work for the three porphyrins tested, it was not shown any changes on the type of mechanisms followed in the course of the photodynamic reaction, even for Tri-Py$^+$-Me-PF for which the concentration used was ten times lower than those used for the other two PS. It is well documented that type I pathway mostly depends on the type and concentration of PS (Korycka-Dahl and Richardson, 1978). However, for the three PS used in this study, the type II pathway is clearly the most implicated in the bacterial oxidation and, consequently, the type and

concentration of PS do not affect the course of the reaction. As the type II reaction is mostly dependent on the solubility and concentration of oxygen (He et al., 1998; Song et al., 1999) and the solubility of the three porphyrins and the concentration of oxygen are similar during the laboratorial experiments for all PS, no difference is observed among them during the irradiation period.

As shown by Alves et al. (2008), the use of stable bioluminescent bacteria allows following the progress of the PI process with real-time results. The bacterial bioluminescent method is also a simple, fast, cost-effective and sensitive way to evaluate which mechanism is responsible for the bacterial inactivation during the photodynamic process (Alves et al., 2008). The relationship between viable counts and the bioluminescence signal is similar in absence and in presence of the PS showing that it is not toxic to the recombinant bioluminescent *E. coli* and does not affect the relationship between bacterial growth and bioluminescence (Alves et al., 2008). Furthermore, the results obtained in this study with the controls indicate that the viability of bioluminescent strain is not affected by the porphyrin after 270 minutes of incubation in the dark (porphyrin dark control) or by light irradiation nor by the inhibitors at the tested concentrations (inhibitor light and dark controls). The bioluminescent *E. coli* is only affected by irradiation in the presence of the sensitizer indicating that the decrease in bacterial viability is due to the PI process.

CONCLUSION

It can be concluded that singlet oxygen (type II mechanism) plays the most important role on the PI process of the tested bioluminescent *E. coli* by Tri-Py^+-Me-PF, Tetra-Py^+-Me and Tri-SPy^+-Me-PF derivatives. The employment of scavengers to evaluate which pathway(s) is(are) involved in the photodynamic process is an adequate and simple approach. However, it has to be taken into account that scavengers must be chosen considering the chemical structure of the photosensitizer.

CHAPTER 3

ANTIMICROBIAL PHOTODYNAMIC THERAPY: STUDY OF BACTERIAL VIABILITY RECOVERY AND POTENTIAL DEVELOPMENT OF RESISTANCE AFTER TREATMENT

ANTIMICROBIAL PHOTODYNAMIC THERAPY: STUDY OF BACTERIAL RECOVERY VIABILITY AND POTENTIAL DEVELOPMENT OF RESISTANCE AFTER TREATMENT

ABSTRACT

Antimicrobial photodynamic therapy (aPDT) has emerged in the clinical field as a potential alternative technique to antibiotics drugs to treat microbial infections. At the moment, it is still not known any microbial viability recovery nor any resistance mechanism against it. In photodynamic therapy it is used a non-toxic photosensitizer that may be activated by low doses of visible light of an appropriate wavelength and generate reactive oxygen species (singlet oxygen and free radicals) which are toxic to target cells. In this work, we aim to test if bacteria can recover their metabolism after photodynamic inactivation and to study the possible development of resistance mechanisms after several treatments. The tricationic porphyrin Tri-Py^{+}-Me-PF will be used as photosensitizer and the bioluminescent bacteria *Vibrio fischeri* and recombinant *Escherichia coli* as bacterial models. To determine the recovery of bacterial activity after treatment, 5.0 μM of Tri-Py^{+}-Me-PF were added to individual bacterial suspensions and the samples were irradiated with white light (4 mW cm^{-2}) during 270 minutes. After treatment, the samples were protected from light with aluminium foil and maintained at 25°C under stirring (100 rpm). Aliquots of the samples were collected 24, 48, 72 and 168 hours after the photodynamic treatment and the bioluminescence signal was measured in a luminometer. To assess the development of resistance in bacterial cells after treatment, bacterial suspensions were exposed to white light for 25 minutes, with 5.0 μM of Tri-Py^{+}-Me-PF and plated on appropriate media. After the first irradiation period, three surviving colonies were collected from the plate and each of them was suspended in PBS. The three bacterial suspensions were exposed to visible light using an identical irradiation protocol. This procedure was repeated for ten times for each bacterium. The obtained results suggest that aPDT using Tri-Py^{+}-Me-PF represents a promising approach to efficiently destroy bacteria since after a single treatment these microorganisms do not recover their viability and after ten generations of partially photosensitized cells both bacteria do not develop resistance to the photodynamic process.

Keywords Cationic porphyrins; antimicrobial photodynamic therapy; bacterial resistance; bacterial viability; bioluminescence; *Vibrio fischeri; Escherichia coli*

INTRODUCTION

The use of antibiotics to destroy selectively microorganisms (MO) represents one of the most revolutionary progresses made in scientific medicine, resulting in the treatment and sometimes complete eradication of earlier incurable diseases (Tunger et al., 2000; Jori and Brown, 2004). It might have been supposed that microbiologically-based disease at the beginning of the twenty first century would have been reduced to a level that no longer had a serious impact on human health. However, bacteria have developed resistance mechanisms against antimicrobial drugs which were previously highly effective. Besides, bacteria replicate very rapidly and a mutation that helps a MO to survive in the presence of an antibiotic will quickly become predominant in the microbial

population (Hamblin and Hasan, 2004; Jori and Brown, 2004). Due to resistance to all ß-lactam antibiotics, the glycopeptid antibiotic vancomycin was remained as last line of defense against Gram-positive bacteria. However, methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *enterococci* are resistant species that are causing much concern at present (Cunha, 1998). There is an urgent need for the development of novel, convenient, non-resistant and inexpensive measures for fighting microbial diseases (Malik et al., 1990; Wainwright, 1998; Jori and Brown, 2004).

Antimicrobial photodynamic therapy (aPDT) represents a potential alternative methodology to inactivate microbial cells (Taylor et al., 2002; Winckler, 2007; Caminos et al., 2008) and has already showed to be effective *in vitro* against bacteria, fungi, viruses, and protozoa (Merchat et al., 1996b; Wainwright, 1998; Bonnett, 2000; Jemli et al., 2002; Wainwright, 2004; Alves et al., 2008; Costa et al., 2008; Alves et al., 2009; Oliveira et al., 2009). The aPDT approach is based on the photodynamic therapy concept that comprises the action of three components: a photosensitizing agent (PS), light of an appropriate wavelength (artificial light or sunlight) and the presence of oxygen (Wainwright, 1998; Bonnett, 2000; Wainwright, 2000; Jori and Brown, 2004; Jori et al., 2006). Two oxidative mechanisms of photoinactivation (PI) are considered to be implicated in the inactivation of the target cells. The type I pathway involves electron/hydrogen atoms-transfer reactions from the PS triplet state with the participation of a substrate to produce radical ions while the type II pathway involves energy transfer the PS triplet state to molecular oxygen to produce excited-state singlet oxygen ($^1O_2$) (Wainwright, 1998; De Rosa and Bentley, 2000; Hamblin and Hasan, 2004; Donnelly et al., 2008; Calin and Parasca, 2009). Both of these processes lead to highly toxic reactive oxygen species (ROS) such as $^1O_2$ and free radicals, able to irreversibly alter cells' vital components resulting in oxidative lethal damage (De Rosa and Crutchley, 2002; Ergaieg et al., 2008). The principal advantages of aPDT are the MO non-target specificity, the few side effects, the prevention of the regrowth of the MO after the treatment and the lack of development of resistance mechanisms due to the mode of action and type of biochemical targets (multi-target process) (Jori et al., 2006; Winckler, 2007).

The photodynamic activity can mainly produce changes in the cytoplasmatic membrane and damages the DNA (Hamblin and Hasan, 2004). The damages to the cytoplasmatic membrane can involve leakage of cellular contents or inactivation of membrane transport systems and enzymes (Li et al., 1997; Mettath et al., 1999). Some damages produced in the DNA chain can be repaired by the action of DNA repairing systems (Imray and MacPhee, 1973). However, it was concluded that although DNA damage occurs it cannot be the main cause of bacterial cell photodynamic inactivation (Hamblin and Hasan, 2004; Durantini, 2006), since *Deinococcus radiodurans*, which is known to have a very efficient DNA repair mechanism, is easily killed by aPDT (Schafer et al., 1998).

Although various studies investigated the possible recovery of bacterial infections in animal models (*in vivo*) (Orenstein et al., 1997; Gad et al., 2004b; Lambrechts et al., 2005b), there are not published studies that tested, *in vitro*, the possible viability recovery after an aPDT treatment. Moreover, despite various authors refer that resistance to aPDT is unlikely because the mechanism of killing is non-specific, with ROS causing damage of diverse bacterial structures (Ito and Kobayashi, 1977; Bagchi and Sreeradha, 1989; Ehrenberg et al., 1993; Bhatti et al., 1998; Carré et al., 1999; Maisch et al., 2004; Omar et al., 2008; Cassidy et al., 2009), only some studies were made to determine if bacterial resistance occurs after several treatments of aPDT. Cell wall structures and membranes are the main target of photodynamic therapy drugs, and for this reason the drugs do not necessarily need to enter the cell. Specific and proper adhesion to these structures suffices for light-activated destruction of the target cell. Thus target cells have no chance to develop resistance by stopping uptake, increasing metabolic detoxification or increasing export of the drug (Winckler, 2007). The investigations in aPDT are more focused to the identification of new PS that kill rapidly and efficiently the MO at low costs and to determine the way of inactivation of those PS. However, and regarding the emergence of bacterial resistance to antibiotics, it is important to control the process of PI in terms of resistance development. Lauro et al. (2002) investigated the selection of resistant bacterial strains in *Peptostreptococcus micros* and *Actinkobacillus actinomycetemcomitans* after repeated photosensitization of surviving cells with the

porchycene-polylysine conjugates 2,7,12,17-tetrakis(2-methoxyethyl)-9-glutaramidoporphycene (GlamTMPn) and 2,7,12,17-tetrakis(2-methoxyethyl)-9-*p*-carboxybenzyloxyporphycene (BOHTMPn). The results obtained by this group showed that the photosensitization of *P. micros* and *A. actinomycetemcomitans* by both PS induced no appreciable development of resistance in partially inactivated bacterial cells. The efficiency of photokilling underwent no change in ten subsequent irradiation sessions, even though cells which were damaged in a previous treatment were cultivated and re-exposed to porphyene and light (Lauro et al., 2002). Pedigo et al. (2009) determined the possible development of bacterial resistance to aPDT after several treatments in antibiotic sensitive (MSSA) and resistant strains (MRSA) of *S. aureus* and antibiotic sensitive *Escherichia coli*. Bacteria were exposed to repetitive aPDT treatments using methylene blue as PS and 670 nm illumination from a non-thermal diode laser. The parameters were adjusted such that kills were lowest than 100% so that surviving colonies could be employed for succeeding exposures. No significant difference in killing of *E. coli* was observed through eleven repeated exposures. Similar results were seen using MSSA and MRSA, for which kill rate did not significantly differ from over twenty five repeated exposures (Pedigo et al., 2009). Jori et al. (unpublished data) determined that up to five consecutive generations of extensively photoinactivated MRSA (ca. 90%) show essentially identical degrees of sensitivity to phthalocyanine photosensitization (Jori and Coppellotti, 2007). Although the known studies indicate that bacterial resistance to aPDT is unlikely, it is an important parameter to be evaluated when a new PS is considered for aPDT.

To monitor the possible development of bacterial resistance to aPDT, faster methods are needed as alternative to laborious conventional methods of diluting, plating, overnight incubation and time-consuming counting of colony-forming units (CFU) (Simon et al., 2001; Vesterlund et al., 2004; Demidova and Hamblin, 2005; Alves et al., 2008). The employment of bacterial bioluminescence method is considered to be a rapid, sensitive and cost-effective choice (Francis et al., 2001; Hamblin et al., 2002; Vesterlund et al., 2004; Alves et al., 2008) to monitor the possible development of resistance after several treatments to aPDT, and can be measured directly, continuously and non-destructively in

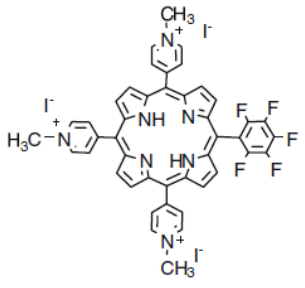
high-throughput screening or continuous-culture models (Beard et al., 2002). A strong correlation between bioluminescence and viable counts was demonstrated in experimental systems (Marincs, 2000; Rocchetta et al., 2001; Alves et al., 2008), where the light output reflects the actual metabolic rate.

The aim of this study is to determine if bacterial cells can recover their activity after photodynamic treatment and to investigate the possible development of resistance to aPDT after various treatments. To achieve these goals, two bioluminescent Gram-negative bacteria were tested (*Vibrio fischeri* and recombinant *Escherichia coli*), using a *meso*-substituted tricationic porphyrin derivative as PS and the bacterial bioluminescent method to evaluate bacterial activity.

MATERIALS AND METHODS

PHOTOSENSITIZER

In this work it was used the tricationic porphyrin derivative 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py^{+}-Me-PF), that was prepared in two steps according to the literature (Tomé et al., 2004). In the first step, the neutral porphyrin was synthesized by crossed Rothemund reactions using pyrrole and the adequate benzaldheydes at reflux in acetic acid and nitrobenzene. The resulting porphyrin was separated by column chromatography (silica) and pyridyl groups quaternized by reaction with methyl iodide. The porphyrin was purified by crystallization from chloroform/methanol/petroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. A stock solution of 500 µM of the porphyrin (dissolved in DMSO) was prepared and then maintained at 4°C.



Tri-Py^{+}-Me-PF

Figure 17 – Structure of 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide.

BACTERIAL STRAINS AND GROWTH CONDITIONS

The bacterial strains used in this work were a recombinant bioluminescent strain of *E. coli* prepared in a previous work (Alves et al., 2008) and the bioluminescent marine bacterium *Vibrio fischeri* ATCC 49387. Both *E. coli* and *V. fischeri* were stored at -80°C in 10% of glycerol.

Before each assay, an aliquot of *V. fischeri* was aseptically plated on tryptic soy agar (TSA, Merck) complemented with 3% of NaCl (because of the osmotic pressure required to natural light emission to occur) and grown for one day at 25°C. Next, one colony was aseptically inoculated on Luria-Bertani broth with saline medium (LBS; 10 g of tryptone, 5 g of yeast extract and 30 g NaCl per liter; pH 7.5) (Geske et al., 2007) and grown for one day at 25°C under stirring (100 rpm). Afterwards, an aliquot of this culture was subcultured in 30 mL of LBS and grown overnight at 25°C, 100 rpm stirring. The same procedure was carried out with *E. coli*, however, this bacterium contains two plasmids that confer resistance to two antibiotics: ampicilin (Amp) and chloramphenicol (Cm). Consequently, *E. coli* was aseptically plated on TSA with 100 mg mL^{-1} of Amp and 25 mg mL^{-1} of Cm and grown for one day at 37°C. Next, one colony was aseptically inoculated on tryptic soy broth (TSB, Merck) with both antibiotics and grown for one day at 25°C under stirring (100 rpm). Then, an aliquot of this culture was subcultured in 30 mL of TSB with Amp and Cm and grown overnight at 25°C, 100 rpm stirring.

BIOLUMINESCENCE VERSUS CFU

To evaluate the correlation between the CFU and the bioluminescence signal of *V. fischeri*, two assays were carried out in dark conditions, with and without porphyrin (Tri-Py^{+}-Me-PF). Two suspensions were prepared from an overnight culture of *V. fischeri*, diluting the culture (1:10) in fresh phosphate buffered saline with 3% of NaCl (PBS with 3% of NaCl: 30 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter; pH 7.4) to a final concentration of 10^7 CFU mL^{-1}. In one of these bacterial suspensions, an appropriate volume of porphyrin was added to achieve a final concentration of 5.0 µM, followed by a dark incubation during 4h at 25-28°C under stirring. Next, both suspensions were serially diluted (10^{-1} - 10^{-7}) in PBS with 3% of NaCl. The non-diluted (10^0) and the diluted aliquots were plated in TSA with 3% of NaCl (100 µL) and, simultaneously, were read on a

luminometer (500 µL) (TD-20/20 Luminometer, Turner Designs, Inc., USA) to determine the bioluminescence signal.

The correlation between the CFU and the bioluminescence signal of *E. coli* was determined in a previous study (chapter 2 of this thesis). The procedure was the same done with *V. fischeri*, however, the suspensions were prepared in PBS without the addition of NaCl (PBS; 8 g NaCl, 0,2 g KCl, 1,44 g Na_2HPO_4 and 0,24 g KH_2PO_4 per liter; pH 7.4) and pour plated in TSA without NaCl.

Both experiments were done in duplicate and the results were averaged.

aPDT RECOVERY STUDY

A photodynamic inactivation assay was done in order to determine if bacterial cells can recover their metabolism after an effective treatment. For this purpose, cultures of *V. fischeri* and *E. coli* were grown overnight and diluted in PBS, supplemented with 3% of NaCl in the case of *V. fischeri*, to a final concentration of 10^7 CFU mL^{-1}. These bacterial suspensions were equally distributed in 600 mL sterilized and acid-washed beakers. Afterwards, the appropriate volume of Tri-Py^+-Me-PF was added to achieve a final concentration of 5.0 µM (total volume in the beakers was 15 mL). Light and dark controls were included in all experiments. The samples were protected from light with aluminum foil and incubated for 10 min under 100 rpm stirring (25-28°C) to promote the porphyrin binding to bacterial cells. Then, the mixtures were exposed to white light with a fluence rate of 4 mW cm^{-2} for 270 minutes (corresponding to a light fluence of 64.8 J cm^{-2}) under 100 rpm (25-28°C). Aliquots of treated and control samples were collected at time 0 and after 15, 30, 45, 60, 90, 180 and 270 min of light exposure and the bioluminescence signal was measured in the luminometer. After 270 min of irradiation, when all bacteria were inactivated to the detection limit of the method, the samples were protected from light with aluminum foil and maintained under stirring at 25-28°C. Aliquots of treated and control samples were collected after 24, 48, 72 and 168 hours post-treatment and the bioluminescence signal was measured in the luminometer.

Both experiments were done in duplicate and the results were averaged.

aPDT RESISTANCE STUDY

In order to assess the possible development of resistance in bacterial cells after photosensitized inactivation, cultures of *V. fischeri* and *E. coli* grown overnight were centrifugated at 10 000 *g* during 15 minutes to remove dead cells and residues of culture medium, and the cells thus obtained were ressuspended in PBS, supplemented with 3% of NaCl in the case of *V. fischeri*. Bacterial suspensions (10^7 CFU mL^{-1}) were equally distributed in 600 mL sterilized and acid-washed beakers and the suitable volume of Tri-Py^+-Me-PF was added to achieve a final concentration of 5.0 µM (total volume was 10 mL per beaker). The samples were protected from the light with aluminum foil and incubated for 10 min under 100 rpm stirring (25°C) to promote the porphyrin binding to bacterial cells. Afterwards, the mixtures were exposed to white light with a fluence rate of 4 mW cm^{-2} (under stirring at 25-28°C) to reach less than 100% of bacterial PI, around 1 log unit of surviving bacteria (25 minutes for both bacteria). At the end of each treatment, an aliquot of both samples was plated on TSA and the plates were incubated at 25°C in the case of *V. fischeri* and at 37°C for *E. coli*. Three colonies surviving after the first irradiation period were collected from the respective plate and each one was centrifugated at 10 000 *g* (15 minutes), ressuspended in PBS and inoculated on the suitable liquid medium. Subsequently, the bacterial suspensions were incubated in the dark whit the PS and exposed to visible light using an identical irradiation protocol that was repeated for ten times for each bacterium. Before each assay, the optical densities of cultures of *V. fischeri* and *E. coli* were controlled and monitored to 0.5 and 1.3 (660 nm), approximately. The PI efficiency was expressed as log N_0/N, where N_0 and N represent the bioluminescence signal before and after the irradiation, respectively (Lauro et al., 2002).

IRRADIATION CONDITIONS

The studies were carried out by exposing the samples to white light (PAR radiation, 13 lamps OSRAM 21 of 18 W each one, 380–700 nm) with a fluence rate of 4 mW cm^{-2} (measured with a radiometer LI-COR Model LI-250). As *V. fischeri* emits light at temperatures below 30°C (Scheerer et al., 2006), the samples were placed on a tray with water in order to maintain the samples at a constant temperature (25-28°C).

RESULTS

BIOLUMINESCENCE VERSUS CFU

It was observed (Figure 18) a linear correlation between viable counts and the bioluminescence signal of overnight cultures of the bioluminescent marine bacterium *V. fischeri* and of the recombinant bioluminescent *E. coli.* These correlations are similar in the presence and in the absence of Tri-Py^{+}-Me-PF.

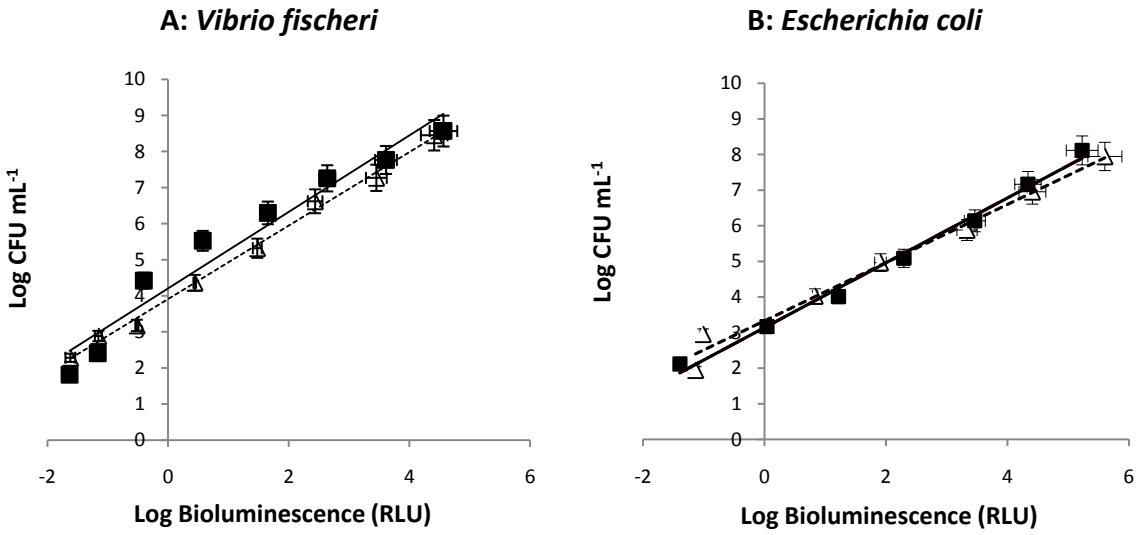


Figure 18 - Relationship between the bioluminescence signal and viable counts of overnight cultures of bioluminescent marine bacterium *Vibrio fischeri* serially diluted in PBS with 3% of NaCl (A) and recombinant bioluminescent *Escherichia coli* serially diluted in PBS (chapter 2 of this thesis) (B) . Viable counts are expressed in CFU mL^{-1} and bioluminescence in relative light units (RLU). Each value represents mean ± standard deviation of two independent experiments (--△-- bacterial suspension in the absence of PS, —■— bacterial suspension with 5.0 µM of Tri-Py^{+}-Me-PF incubated in the dark).

aPDT RECOVERY STUDY

The ability of *V. fischeri* and *E. coli* cells to recover their metabolic capacities after a photodynamic treatment with 5.0 µM of Tri-Py^{+}-Me-PF is represented in Figure 19 and Figure 20, respectively. After 270 minutes of irradiation, it was obtained a reduction of 5.1 log units on the bioluminescence signal of *V. fischeri* and 6.4 log units for bioluminescent *E. coli*. Moreover, light and dark controls results show that the viability of these bacteria was neither affected by irradiation itself nor by the PS in dark conditions (Figures 19A and 20A). This indicates that the reductions obtained on cell viability after irradiation of the treated samples are due to the photosensitizing effect of the porphyrin.

After one week of incubation in dark conditions (Figures 19B and 20B), it was observed that the bioluminescence signal of treated samples of *V. fischeri* and *E. coli* was the same during all period of incubation (≈ log -1.5 RLU for both *V. fischeri* and *E. coli* cells). It was also observed a decrease on the bioluminescence signal of light and dark controls of these bacteria.

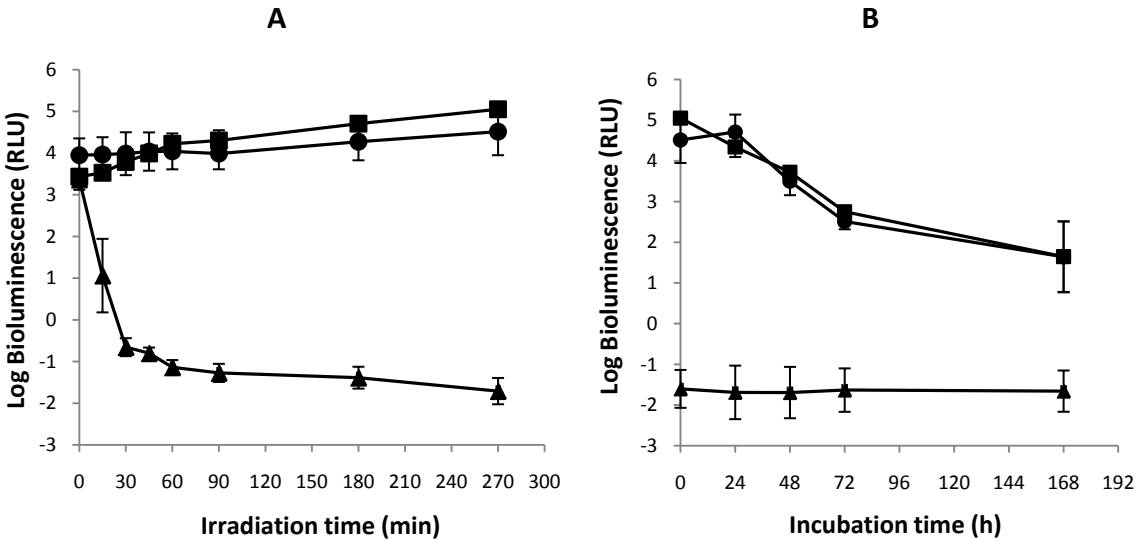


Figure 19 – Logaritmic reduction of *V. fischeri* treated with porphyrin Tri-Py^{+}-Me-PF at 5.0 µM after 15, 30, 45, 60, 90, 180 and 270 minutes of irradiation at 4 mW cm^{-2} (A). Bioluminescence signal of *V. fischeri* after 24, 48, 72 and 168 hours of the photodynamic treatment (B). Each value represents mean ± standard deviation of two independent experiments (-■- Tri-Py^{+}-Me-PF dark control, -●- *V. fischeri* light control, -▲- Tri-Py^{+}-Me-PF 5.0 µM).

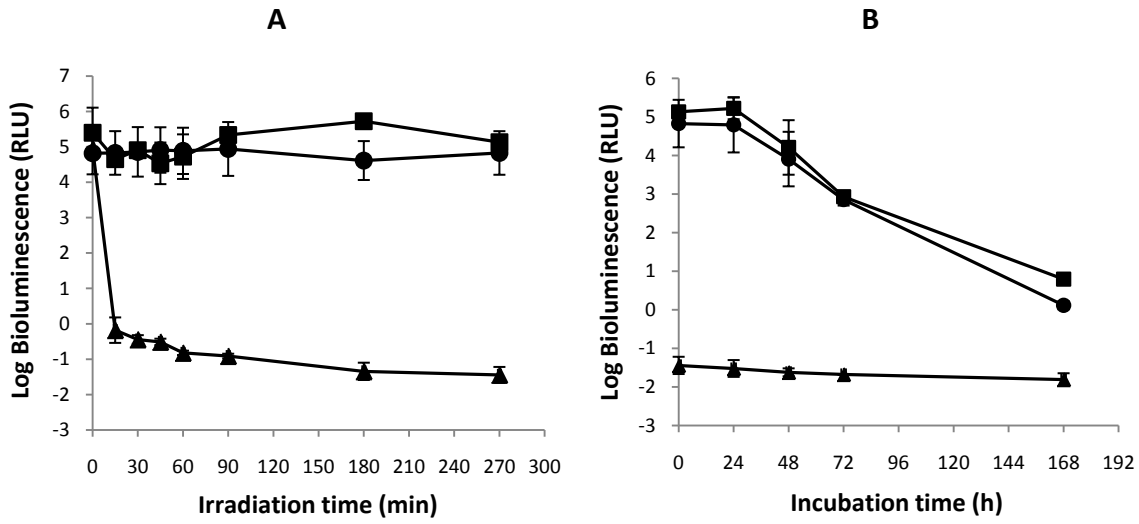


Figure 20 – Logaritmic reduction of *E. coli* treated with porphyrin Tri-Py^{+}-Me-PF at 5.0 µM after 15, 30, 45, 60, 90, 180 and 270 minutes of irradiation at 4 mW cm^{-2} (A). Bioluminescence signal of *E. coli* after 24, 48, 72 and 168 hours of the photodynamic treatment (B). Each value represents mean ± standard deviation of two independent experiments (-■- Tri-Py^{+}-Me-PF dark control, -●- *E. coli* light control, -▲- Tri-Py^{+}-Me-PF 5.0 µM).

aPDT RESISTANCE STUDY

The repeated treatments of aPDT to determine the possible development of resistance to Tri-Py^{+}-Me-PF porphyrin in partially inactivated *V. fischeri* and *E. coli* cells are represented in Figure 21A and 21B, respectively. It was observed that the efficiency of PI underwent no reduction in ten subsequent irradiations to destroy 99.99% of *V. fischeri* and *E. coli* cells.

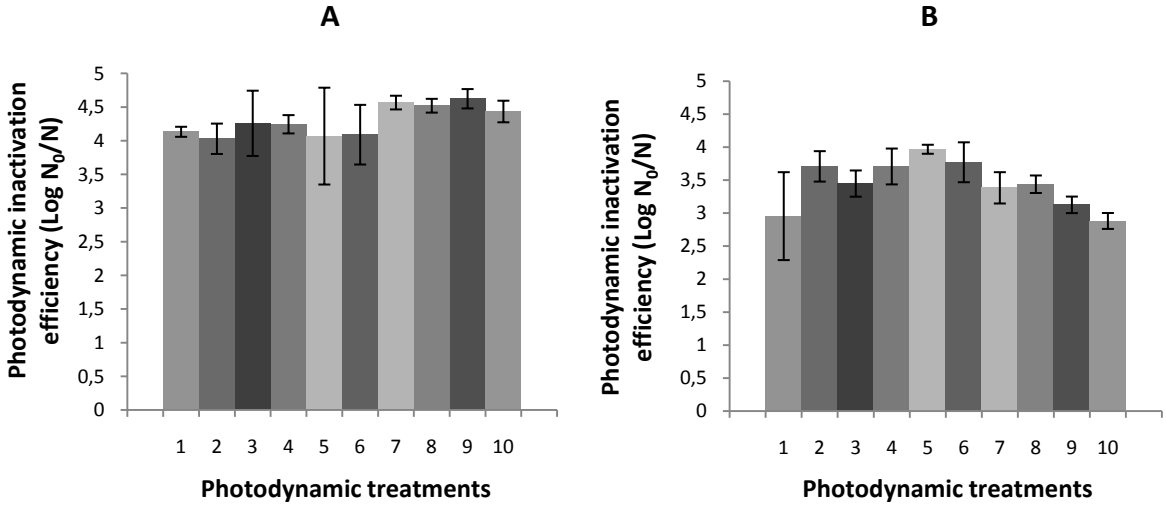


Figure 21 – Photodynamic inactivation efficiency of ten consecutive generations of a) *V. fischeri* and b) *E. coli* by 5.0 µM of Tri-Py^{+}-Me-PF after 25 minutes of irradiation at 4 mW cm^{-2}. N_0 and N represent, respectively, the bioluminescence signal before and after the irradiation (Lauro et al., 2002). Each value represents mean ± standard deviation of three independent experiments.

DISCUSSION

Microorganisms have adopted a large variety of mechanisms to increase their resistance to antimicrobial drugs. These mechanisms comprise a thickening of their outer wall, encoding of new proteins which prevent the penetration of drugs, and onset of mutants deficient in those porin channels allowing the influx of externally added chemicals (Harder et al., 1981; Roland et al., 1994; Boyle-Vavra et al., 2001). The emergence of antibiotic resistance by pathogenic MO has led to the search of efficient alternative methods for which mechanisms of resistance must not occur (Cassell and Mekalanos, 2001; Hamblin and Hasan, 2004). The aPDT represents a potential approach to inactivate pathogenic MO (Taylor et al., 2002; Caminos et al., 2008) and has already showed to be efficient against bacteria, yeasts, viruses, and protozoa (Merchat et al.,

1996b; Wainwright, 1998; Bonnett, 2000; Jemli et al., 2002; Wainwright, 2004; Alves et al., 2008; Costa et al., 2008; Alves et al., 2009; Oliveira et al., 2009). The main advantages of aPDT are the MO non-target specificity, the few side effects, the prevention of the regrowth of the MO after treatment and the potential lack of development of resistance mechanisms due to the mode of action and type of biochemical targets (multi-target process) (Jori et al., 2006; Winckler, 2007). In fact, as the main target of aPDT are cell wall structures and membranes, the PS do not need to enter in the cell, having no chance to develop resistance by stopping uptake, increasing export of the PS or increasing metabolic detoxification (Winckler, 2007).

The results of this study shown that bacteria do not recover their metabolic capacities after 270 minutes of irradiation at 4 mW cm^{-2} using the Tri-Py^{+}-Me-PF porphyrin. It was observed that when the samples are maintained during one week, in dark conditions, after the photodynamic treatments, the bioluminescence signal of both tested bacteria remains the same during all period of incubation (≈ log -1.5 RLU for both *V. fischeri* and *E. coli* cells). Dark and light controls showed a decrease on the bioluminescence signal, maybe as a result of the accumulation of toxic products resulting from metabolism and also maybe due to the lack of nutrients. These results are in agreement with the data obtained by Costa et al. (unpublished data) that observed that after a treatment with Tri-Py^{+}-Me-PF porphyrin, it was not verified any recovery of T4-like bacteriophages following one week of incubation in dark conditions (Costa et al., unpublished data).

It was also observed that the results obtained in this study corroborate the literature (Lauro et al., 2002; Jori and Coppellotti, 2007; Pedigo et al., 2009) in relation to the potential lack of microbial development of resistance mechanisms after the photodynamic process. It was demonstrated that bacterial resistance do not occur after ten repeated photosensitization processes using the bioluminescent marine bacterium *V. fischeri* and the bioluminescent recombinant *E. coli*. In fact, it was not observed any considerable reduction in the efficiency of photosensitization of *V. fischeri* and *E. coli* after repetitive photosensitization sessions of 25 minutes with 5.0 μM of Tri-Py^{+}-Me-PF. If bacterial resistance occurred, it was expected that the time of irradiation increased from

each treatment to reach the 99.99% of photosensitized cells. Various authors affirmed that stationary phase cultures show a certain degree of resistance to the PS (Nitzan et al., 1989; Bhatti et al., 1998). Others authors refer, however, that bacteria susceptibility to PS are independent from the growth phase (Gad et al., 2004a; Banfi et al., 2006). In this study, the cultures were used at the same growth phase (stationary growth phase) for all assays. As the bacterial colony has been aseptically removed from the plate and ressuspended in PBS, the cellular density obtained after the colony ressuspension could be different. To avoid differences in the efficiency of PI due to different bacterial density, this parameter was controlled in all the experiments by measuring the optical density of the bacteria suspension before each assay.

It can be concluded that the promising photosensitizer Tri-Py^{+}-Me-PF is able to destroy efficiently Gram-negative bacteria, after the photodynamic treatment, without the recovery of bacterial viability. It was also confirmed that the bacteria photosensitized by this photosensitizer do not develop resistance mechanisms against the photodynamic process.

CHAPTER 4
DISCUSSION

Photosensitized oxidations can take place by two mechanisms known as type I and type II mechanisms. The type I pathway involves the initial interaction between the excited PS and the substrate by hydrogen-atom or electron transfer resulting in the production of free radicals. The type II mechanism takes place between molecular oxygen and the excited PS to produce singlet oxygen. All of these ROS formed in both pathways are able to irreversibly alter the cells' vital components resulting in oxidative lethal damage of the target cell (Wainwright, 1998; De Rosa and Crutchley, 2002; Hamblin and Hasan, 2004). It is important to know which ROS species are generated in order to improve the design of the PS and to decide about the best conditions for microbial photoinactivation. Although there are some studies that investigate which $^1O_2$ or free radicals pathways are involved in the photoinactivation reaction, only few studies were done using MO, namely bacterial cells, and scavengers. Typical type I reactions at the bacterial cytoplasmatic membrane include the abstraction of allylic hydrogens from unsatured molecules such as phospholipids. The radical species thus formed may undergo reaction with oxygen to yield the lipid hydroperoxide leading to loss of fluidity and increased permeability (Korytowski et al., 1992; Wainwright, 1998). Singlet oxygen also reacts with biomolecules involved in the maintenance and structure of the cell wall/membrane such as phospholipids, peptides and sterols (Wainwright, 1998). Nucleic acids are known to react mainly through guanosine residues (Foote, 1990). Some damages produced in the DNA chain can be repaired by the action of DNA repairing systems (Imray and MacPhee, 1973). However, some authors concluded that although DNA damage occurs, it may not be the main cause of bacterial cell death (Hamblin and Hasan, 2004). As the main targets of ROS are cell wall structures and membranes, the PS does not necessarily need to enter the cell to cause cell death. A specific and a proper adhesion of the PS to these structures is sufficient for the light-activated destruction of the target cell. Consequently, target cells have no chance to develop resistance by stopping uptake, increasing metabolic detoxification or increasing the export of the PS (Winckler, 2007).

According to the results obtained in this work, singlet oxygen-mediated reaction (type II mechanism) plays the most important role in the process of photoinactivation of

the strain of *E. coli*. Despite type II mechanism is the predominant reaction in the photodynamic inactivation, free radicals-mediated reactions are also produced simultaneously, contributing to the bacterial photoinactivation but to a much smaller extent. These data are in agreement with the literature where type II photoprocess is generally accepted as the major pathway in photooxidative microbial cell damage (Wainwright, 1998; Maclean et al., 2008; Omar et al., 2008) and that type I mechanism can also contribute in a minor way to the photodynamic inactivation (Nitzan et al., 1989; Müller-Breitkreutz et al., 1995; Abe et al., 1997; Wong et al., 2005; Ergaieg et al., 2008). Ergaieg et al (2008) studied the mechanism involved in the phototoxicity of *meso*-tetra (*N*-methyl-4-pyridyl) porphyrin tetra-tosylate (TMPyP) on *E. hirae* and *E. coli* using specific scavengers and quenchers of ROS. The group verified that using the singlet oxygen quenchers sodium azide, histidine and *β*-carotene, reductions on the photoinactivation activity of *E. hirae* and *E. coli* were obtained. Furthermore, using the free radical scavengers superoxide dismutase, catalase and DMSO they also achieved an decrease on the activity of TMPyP concluding that both type I and type II reactions play important roles on the process of PI of TMPyP (Ergaieg et al., 2008). The mechanism of *S. aureus* inactivation by deuteroporphyrin (DP) was investigated by Nitzan et al. The light-activated DP (10 μM mL^{-1}) reduced the viability of the culture to less than 1%, while the singlet oxygen quenchers methionine, Trp, and 1,4-diazabicyclo-2,2,2-octane (DBCO) used as provided approximately 60% protection. Propylgallate (free radical scavenger) also conferred 60% of protection. The presence of a singlet oxygen quencher and propylgallate provided almost a complete protection from the photoinactivation (96%), indicating that *S. aureus* photoinactivation by DP is mediated by both singlet oxygen and hydroxyl free radicals (Nitzan et al., 1989).

In this work, when sodium azide was used at 100 mM, reductions on the PI of the porphyrins Tri-Py^{+}-Me-PF, Tetra-Py^{+}-Me and Tri-SPy^{+}-Me-PF were obtained after 270 minutes of irradiation at 4 mW cm^{-2}. Porphyrin Tri-Py^{+}-Me-PF (0.5 μM) was the most effective PS (reduction of 5.1 log units for Tri-Py^{+}-Me-PF and reductions of 3.9 and 3.3 log units for Tetra-Py^{+}-Me 5.0 μM and Tri-SPy^{+}-Me-PF 5.0 μM, respectively). The quenching of $^{1}O_2$ by sodium azide is probably due to a charge transfer process in which molecular

oxygen is released after the reaction and, consequently, no oxygen is consumed (Telfer et al., 1994). Moreover, theses reductions are proportional to the $^1O_2$ generation by the PS, Tri-Py$^+$-Me-PF is the PS that generates the highest amount of the $^1O_2$ and Tri-SPy$^+$-Me-PF is the porphyrin that generates the smallest amount of $^1O_2$.

When the free radical scavenger D-mannitol at 100 mM was used, it was observed a small reduction on the PI of all of the PS tested. Reductions of 0.7, 0.2 and 0.7 log units were obtained on the PI of Tri-Py$^+$-Me-PF (0.5 μM), Tetra-Py$^+$-Me (5.0 μM) and Tri-SPy$^+$-Me-PF (5.0 μM), respectively, after 270 minutes of irradiation at 4 mW cm^{-2}. When L-cysteine was used as free radical scavenger, the obtained results were similar from those obtained with D-mannitol for the cationic porphyrins Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me. Reductions of 0.3 and 0.2 log units on the PI of Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me were achieved after 270 minutes of irradiation. However, the results obtained with the tricationic porphyrin Tri-SPy$^+$-Me-PF (5.0 μM) were unexpected and different from those obtained with D-mannitol. At 100 mM of L-cysteine, a high reduction on the PI of the PS was obtained (reduction of 3.2 log units on the activity of Tri-SPy$^+$-Me-PF), reaching similar values than those observed with the $^1O_2$ quencher sodium azide. Taylor and Richardson (1980) affirm that the antioxidant activity of cysteine is due to its sulfhydryl group in biological and other systems (Taylor and Richardson, 1980). According to the chemical structure of Tri-SPy$^+$-Me-PF, it can be supposed that disulfide bridges take place between L-cysteine and Tri-SPy$^+$-Me-PF stopping the activity of the PS and, consequently, the production of ROS. Singlet oxygen generation studies were made in order to understand what happens when L-cysteine is in contact with the PS. The obtained results indicate that the production of $^1O_2$ by the Tri-SPy$^+$-Me-PF porphyrin in the presence of L-cysteine decreases completely, stopping the production of $^1O_2$. These data can explain the fact that when L-cysteine is used, a high reduction on the activity of Tri-SPy$^+$-Me-PF is obtained after 270 minutes of irradiation, as a result of a direct inactivation of the PS by L-cysteine. However not expected, this scavenger also affects the production of $^1O_2$ by the other two porphyrins without sulfhydryl group. But the production of $^1O_2$ is not totally stopped by this scavenger as occurred with Tri-SPy$^+$-Me-PF. Moreover, these reductions on the generation of $^1O_2$ by Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me porphyrins are not relevant

on the study of mechanisms since the data obtained with these PS are different from those obtained with Tri-SPy^{+}-Me-PF, even for Tri-Py^{+}-Me-PF that was used in concentration ten times lower than the other two PS. The effect of D-mannitol on the generation of $^{1}O_2$ was also tested and it was verified that this scavenger does not affect the production of $^{1}O_2$ by all the PS tested. Although the employment of scavengers to evaluate which of the two pathways is involved in the photodynamic process is an adequate and simple approach, these results demonstrate that it is important to have into account that the scavengers must be chosen having in consideration the chemical structure of the photosensitizer.

Min and Boff (2002) affirm that a photosensitized oxidation may change the types of pathway during the course of the reaction due to changes in the concentration of compounds and oxygen changes (Min and Boff, 2002). In our study, it was not detected any change for the three porphyrins tested on the type of mechanisms followed in the course of the photodynamic reaction, even for Tri-Py^{+}-Me-PF. This porphyrin inactivates efficiently and rapidly Gram (+) and Gram (-) bacteria, sewage bacteriophages and bacterial endospores (Alves et al., 2008; Costa et al., 2008; Alves et al., 2009; Oliveira et al., 2009). For this reason, it was necessary to test the mechanism of this PS using a lower concentration comparatively to the other two PS (Costa et al., 2008; Alves et al. 2009; Alves et al. 2009 and unpublish data; Oliveira et al., 2009).

According to Korycka-Dahl and Richardson (1978), the rate of the type I pathway is mainly dependent on the type and concentration of the PS, while the rate of type II pathway is mostly dependent on the solubility and concentration of oxygen (Korycka-Dahl and Richardson, 1978). Maclean et al. (2008) and Feuerstein et al. (2005) demonstrated that oxygen reduction has a negative effect on the photodynamic inactivation process, with the addition of reactive oxygen scavengers significantly reducing the efficacy of the visible light treatment. This was assumed to be due to the failure of the scavengers to efficiently scavenge the fast-binding of the highly reactive $^{1}O_2$ as well as their partially ineffective access to the ROS produced within the cells (Feuerstein et al., 2005; Maclean et al., 2008). In this work and for the three cationic PS tested, the type II mechanism is clearly the main pathway implicated in the bacterial oxidation and, consequently, the

type and concentration of PS did not affect the way of the reaction. Furthermore, the solubility of the three PS and the concentration of oxygen were similar during the laboratorial experiments.

The results obtained with all controls used for the evaluation of the mechanism of photoinactivation showed that the viability of the bioluminescent strain is not affected by the porphyrins after 270 min of incubation in the dark (porphyrin dark control) or by light irradiation nor by the inhibitors at the tested concentrations (inhibitor light and dark controls). The bioluminescent *E. coli* was only affected by the irradiation in the presence of the PS indicating that the decrease in bacterial viability is due to the photoinactivation process.

The major advantages of aPDT are the high target specificity and the prevention of the recovery of microorganisms's viability after the aPDT treatment and the potential lack of development of resistance mechanisms due to the mode of action and the type of biochemical targets (multi-target process) (Jori et al., 2006; Winckler, 2007). The main target of aPDT are cell wall structures and membranes and, for these reasons, the PS do no need to enter in the cell and a specific and proper adhesion to these structures suffices for the light-activated destruction of target cell, with no chance to develop resistance by stopping uptake, increasing export of the PS or increasing metabolic detoxification (Winckler, 2007).

The data obtained in this study demonstrate that bacterial cells do not recover their activity after 270 minutes of irradiation (4 mW cm^{-2}) using the Tri-Py^{+}-Me-PF porphyrin derivative. The bioluminescence signal of both tested bacteria, after the photodynamic treatments, remains the same during all period of incubation (one week in dark conditions), exhibiting very low signals of bioluminescence during this period (≈ log -1.5 RLU for both *V. fischeri* and *E. coli* cells). Light and dark controls register a decline on the bioluminescence signal, probably due to the accumulation of toxic products resulting from metabolism and also maybe to the lack of nutrients. These data are in accordance with the literature where it is hypothesized that MO regrowth must not occur after aPDT treatment (Jori et al., 2006). Moreover, Costa et al. (unpublished data) also verified that

T4-like bacteriophages viability recovery does not occurr after an efficient treatment with the efficient Tri-Py^{+}-Me-PF (Costa et al., unpublished data).

It was also observed in this study that after several generations of photoinactivated cells, *V. fischeri* and *E. coli* do not develop resistance against the porphyrin Tri-Py^{+}-Me-PF. In fact, after ten generations, it was not observed any considerable reduction on the efficiency of photoinactivation of bacteria. If bacterial resistance occurs, it is supposed to increase the time of irradiation to reach 99.99% of photosensitized cells. In this study, it was only necessary 25 minutes to destroy 99.99% of *V. fischeri* and *E. coli* cells during the several treatments of photoinactivated generations. This information is in agreement with the literature where it has not been reported that bacterial resistance occurs after several processes of photodynamic inactivation (Lauro et al., 2002; Jori and Coppellotti, 2007; Pedigo et al., 2009).

Some authors affirm that stationary phase cultures demonstrate a certain level of resistance to the PS (Nitzan et al., 1989; Bhatti et al., 1998). Others refer that bacteria susceptibility to PS is independent from the growth phase (Gad et al., 2004a; Banfi et al., 2006). In this study, the bacterial cultures were used at the same growth phase (stationary growth phase). As one bacterial colony has been aseptically removed from each TSA plate for each independent assay, the cellular density in each colony may have been different. To avoid differences in the PI due to differences of bacterial density, the bacterial growth of these bacteria was controlled in all the experiments.

As shown by Alves et al. (2008), the use of a stable bioluminescent bacterial strain allows following the progress of the photoinactivation process with real-time results having into account that the bioluminescence signal reflects the viable bacterial abundance. The application of this method is considered also a simpler, faster, cost-effective and sensitive way to evaluate which mechanism is responsible for the bacterial inactivation during the photodynamic process, to determine if bacteria recover from photoinactivation and to monitor the possible development of resistance to the ROS produced during the photochemical process. The relationship between viable counts and the bioluminescence signal of *E. coli* is similar in the absence and in the presence of Tri-Py^{+}-Me-PF demonstrating that the PS is not toxic to the recombinant bioluminescent *E.*

coli and it does not affect the relationship between bacterial growth and bioluminescence. The same result was obtained with the bioluminescent marine bacterium *V. fischeri* where the relationship between viable counts and the bioluminescence signal was similar in the presence and in the absence of the PS.

In conclusion, the results show that singlet oxygen-mediated reaction (type II mechanism) plays the most important role on the photoinactivation process of the bioluminescent *Escherichia coli* by the cationic porphyrins Tri-Py^{+}-Me-PF, Tetra-Py^{+}-Me and Tri-SPy^{+}-Me-PF. The use of scavengers to evaluate which pathway is involved in the photodynamic process represents an adequate and simple approach. However, it is essential to have into consideration that the scavengers must be selected having into account the chemical structure of the PS. Finally, the aPDT represents an adequate method to inactivate microorganisms, since bacteria do not recovery their metabolic activity after photoinactivation and bacterial resistance is not developed after several treatments of aPDT using the efficient *meso*-substituted Tri-Py^{+}-Me-PF porphyrin.

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